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13. Abstract *(Maximum 200 Words)* *(abstract should contain no proprietary or confidential information)* VEGF plays an important rc in tumor vascular growth allowing tumor development and metastases. The fusion toxin $VEGF_{121}$/rGel can specifically kill vascular endothelial cells expressing the KDR receptor for VEGF(PNAS 99:7866,2002). Using DNA microarray technology, we have discovered 19 uniq genes upregulated(>5 fold) in endothelial cells treated with $VEGF_{121}$/rGel.Administration $VEGF_{121}$/rGel had a dramatic cytotoxic effect on the tumor growth in both orthotopic and metastatic human breast tumor models. Against the orthotopic model, tumor growth was significantly delayed by~50%. In addition, tumors completely regressed in 3/6 (50%) of treated mice. In the metastatic breast model, treatment with $VEGF_{121}$/rGel reduced both th number and area of lung foci by 58% and 50% respectively. In addition, the number of blo vessels per mm^2 in metastatic foci was 198 ± 37 versus 388 ± 21 for treated and control respectively. Approximately 62% of metastatic colonies from the VEGF/rGel treated group had <10 vessels/colony compared to 23% in the control group. The flk-1 receptor on blood vessel endothelium was intensely expressed on control tumors, but not expressed on treat tumors. Metastatic foci had a 3 fold lower Ki-67 labeling index compared to control tumors. This suggests that $VEGF_{121}$/rGel has impressive antitumor activity in breast cance

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Introduction

Biological studies examining the development of the vascular tree in normal development and in disease states have identified numerous cytokines and their receptors responsible for triggering and maintaining this process *(1-7)*. Tumor neovascularization is central not only to the growth and development of the primary lesion but appears to be a critical factor in the development and maintenance of metastases *(8-12)*. Clinical studies in bladder cancer *(9)* have demonstrated a correlation between micro-vessel density and metastases. In addition, studies of breast cancer metastases by Fox et al. and Aranda et al. *(11-12)* have demonstrated that microvessel count in primary tumors appears to be related to the presence of metastases in lymph nodes and micrometastases in bone marrow.

The cytokine vascular endothelial growth factor-A (VEGF-A) and its receptors Flt-1 and KDR have been implicated as one of the central mediators of normal angiogenesis and tumor neovascularization *(13-20)*. Up-regulation or over-expression of the KDR receptors or the VEGF-A ligand itself have been implicated as poor prognostic markers in various clinical studies of colon, breast and pituitary cancers *(21-23)*. Recently, Padro et al. *(24)* have suggested that both VEGF-A and KDR may play a role in the neovascularization observed in bone marrow during AML tumor progression and may provide evidence that the VEGF/KDR pathway is important in leukemic growth.

For these reasons, there have been several groups interested in developing therapeutic agents and approaches targeting the VEGF-A pathway. Agents which prevent VEGF-A binding to its receptor, antibodies which directly block the KDR receptor itself and small molecules which block the kinase activity of the KDR and thereby block growth factor signaling are all under development *(25-37)*. Recently, our laboratory reported the development of a growth factor

fusion construct of $VEGF_{121}$ and the recombinant toxin gelonin *(38)*. Our studies demonstrated that this agent was specifically cytotoxic only to cells expressing the KDR receptor and was not cytotoxic to cells over-expressing the Flt-1 receptor. In addition, this agent was shown to localize within tumor vasculature and caused a significant damage to vascular endothelium in both PC-3 prostate and MDA-MB-231 orthotopic xenograft tumor models.

The current study seeks to extend our original observations describing the in vitro biological effects of this novel fusion construct and we examined the effects of this agent in both orthotopic and metastatic tumor models.

Progress Report Body:

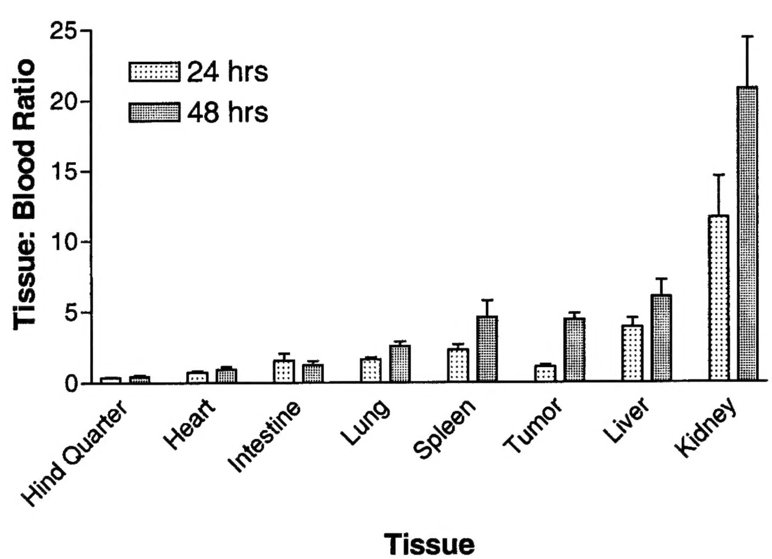
Original SOW:

1. **Establish In Vivo Activity of the $VEGF_{121}$/rGel Fusion Toxin in the MDA-MB231 Tumor Models**

Task 1: Radiolabeling
Numerous methods were employed initially to label the target protein. We settled on using Bolton-Hunter reagent which generated the highest yield of material capable of specific binding to purified, immobilized KDR receptor. Highly purified $VEGF_{121}$/rGel was radiolabeled using ^{125}I with this reagent and the material was adjusted to a specific activity of XXX mg/mCi.
Task 2: Tissue Distribution
Mice bearing orthotopically-placed MDA-MB231 tumors were administered XXXmCi of $VEGF_{121}$/rGel(via iv tail vein). At 24 and 48 hrs after administration, groups of 6 mice were sacrificed and various organs were excised, weighed and counted to determine ^{125}I activity.

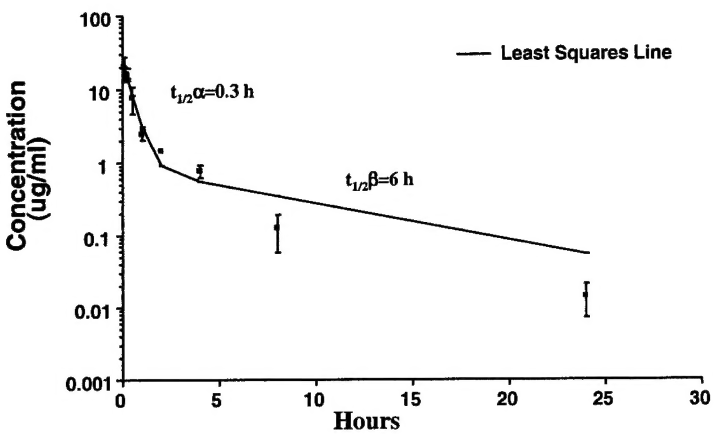
As shown in this figure, the highest concentration of radiolabel was found in kidney> liver>tumor> spleen. At 48 hrs, the tissue:blood ratio in these organs increased particularly for kidney and tumor. The high levels found in kidney may be related to high levels of the flt-1 receptor found in this organ.

Tissue Distribution of ^{125}I-$VEGF_{121}$/rGel in Nude Mice Bearing MDA-MB-231 Xenografts



The pharmacokinetics of $VEGF_{121}$/rGel were additionally described using this radiolabeled material. Balb/c mice were injected with 1 uCi of labeled material and at various times after administration, groups of 3 mice were sacrificed and blood samples were removed and centrifuged. Aliquots of plasma were counted to determine radioactivity and the results were analyzed for conventional pharmacokinetic analyses using conventional mathematical modeling(pK Analyst from MicroMath, Inc). As shown in the figure below, the $VEGF_{121}$/rGel cleared from plasma with initial and terminal half-lives of 0.3 and 6h respectively. Therefore this agent has a relatively long half-life despite the significant uptake in kidney.

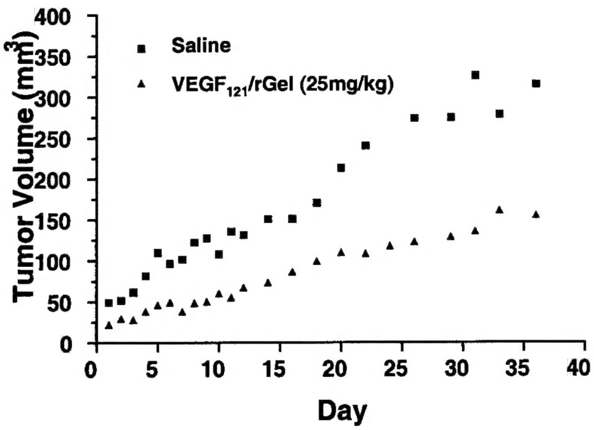
Clearance of $VEGF_{121}$/rGel From Plasma



Task 3: Antitumor Effects-Orthotopic Model

. Given the pharmacokinetic and tissue distribution data described above, we designed a treatement schedule comprised of 5 injections spaced 48 hrs apart(10 day course). Using this schedule, we delineated the Maximum Tolerated Dose as this schedule to be ~25 mg/kg. The effect of $VEGF_{121}$/rGel administration on orthotopically-placed MDA MB231 tumor bearing mice(6 per group) is shown in the Figure below. As shown, treatment significantly retarded tumor growth. In addition, 3/6 mice in the treated group demonstrated complete disappearance of the tumor compared to 0/6 in the saline-treated group.

Effect of $VEGF_{121}$/rGel on Orthotopically Placed MDA-MB-231 Tumor Cells in Nude Mice



Task 3: Antitumor Effects-Metastatic Model

We evaluated the effect of $VEGF_{121}$/rGel fusion toxin treatment on the growth of metastatic MDA-MB-231 tumor cells in nude mice. Tumor cells (0.5×10^6 per mouse) were injected i.v. and 8 days after innoculation, mice (6 per group) were treated 6 times either with $VEGF_{121}$/rGel (100 ug/dose) or free gelonin. Three weeks after treatment, mice were sacrificed and the lungs were harvested and examined. The surface lung foci in the $VEGF_{121}$/rGel – treated mice were reduced by 58 % as compared to gelonin control animals (means were 22.4 and 53.3 for $VEGF_{121}$/rGel and control, respectively; $p<0.05$). The mean area of lung colonies from $VEGF_{121}$/rGel-treated mice was also 50% smaller than control mice (210 ± 37 µm versus 415 ± 10 µm for $VEGF_{121}$/rGel and control, respectively; $p< 0.01$). In addition, the vascularity of metastatic foci as assessed by the mean number of blood vessels per mm^2 in metastatic foci was significantly reduced (198 ± 37 versus 388 ± 21 for treated and control, respectively). Approximately 62% of metastatic colonies from the $VEGF_{121}$/rGel-treated group had fewer than 10 vessels per colony as compared to 23% in the control group. The VEGF receptor(flk-1) was intensely detected on the metastatic vessels in the control but not on the vessels in the $VEGF_{121}$/rGel-treated group.

Metastatic foci present in lung had a 3-fold lower Ki-67 labeling number compared to control tumors. These data strongly suggest that the anti-tumor vascular-ablative effect of $VEGF_{121}$/rGel could be utilized not only for treating primary tumors but also for inhibiting metastatic spread.

Please note that a complete manuscript detailing the impressive anti-metastatic effects of $VEGF_{121}$/rGel in this breast tumor model is attached in the Appendix(The Vascular-Ablative Agent, $VEGF_{121}$/rGel, Inhibits Pulmonary Metastases of MDA-MB-231 Breast Tumors, by Ran et.al)

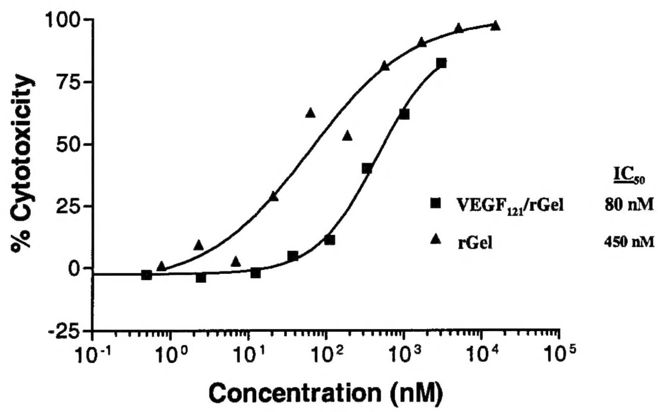
Task 7: Co-Culture of Endothelial Cells and MDA-MB231 Tumor Cells

We utilized the Falcon multi-well culture plates to culture the MDA-MB231 tumor cells. Suspended above these cells, we placed an insert containing log-phase PAE/flk-1 cells. The 2 cell lines are cultured in the same DMEM/F-12 growth media, so no adaptation was required.

Task 8: Studies of $VEGF_{121}$/rGel Effects on Endothelial and Breast Tumor Cells

In the co-culture chambers, the 2 cell lines were treated with various doses of the $VEGF_{121}$/rGel fusion construct targeting the flk-1 receptor on endothelial cells. The MDA-MB231 cells lack this receptor and were not affected by the doses utilized. At various times after drug administration, both cell lines will be harvested, the RNA extracted and analyzed using the Gene Chip as described to assess the impact of treatment on over 7,000 genes including proteins involved in signal transduction, stress response, cell cycle control and metastasis. As shown below, we demonstrate the cytotoxic effects of the fusion construct on endothelial cells. Initial studies demonstrated that the initially-proposed PAE/flk-1 endothelial cells would be utilized, however, Gene Chip analysis showed no hybridization to isolated RNA samples. Troubleshooting demonstrated that there is insufficient homology of the porcine cell RNA to that of the human probes on the Gene Chip, therefore, HUVEC cells were substitued for this phase of study. As shown below, HUVEC cells showed specific cytotoxicity of the fusion construct compared to rGel itself although the magnitude of the differential is lower than that of the PAE/flk-1 cells.

Cytotoxicity of $VEGF_{121}$/rGel on HUVECs



Genes overexpressed(>5X Control) in HUVECs by 24 hr treatment with VEGF121/rGel:

- E Selectin (endothelial adhesion molecule 1)
- Small inducible cytokine A2 (monocyte chemotactic protein 1)
- Tumor necrosis factor, alpha-induced protein 3
- Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha
- Kinesin-like 5 (mitotic kinesin-like protein 1)
- Small inducible cytokine A4
- Jun B proto-oncogene
- Nidogen 2
- Prostaglandin-endoperoxide synthase 2
- Dual specificity phosphatase 5
- Small inducible cytokine subfamily A (Cys-Cys), member 11 (eotaxin)
- Plasminogen activator, urokinase
- Human proteinase activated receptor-2 mRNA, 3'UTR
- Ephrin-B2

Small inducible cytokine A7 (monocyte chemotactic protein 3)

Spermidine/spermine N1-acetyltransferase

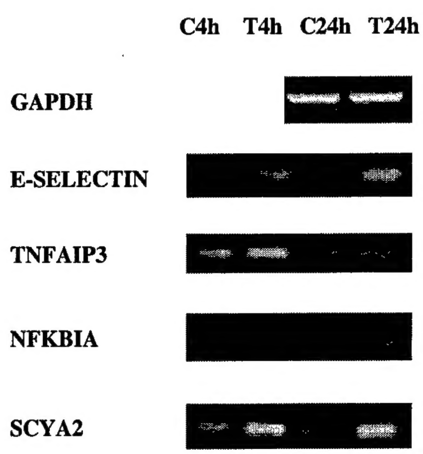
Syndecan 4 (amphiglycan, ryudocan)

Chemokine (C-X-C motif), receptor 4 (fusin)

Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (p105)

All of the genes described above are known in the literature, however their association with cytotoxic events related to toxins such as rGel and activity on endothelial cells was previously unsuspected. To confirm these observations, these studies were repeated and RT-PCR was also employed to more closely assess the timelines for gene modulation. As shown below, our RT-PCR essentially confirmed the observations of the first 5 genes.

Up-regulation of mRNA in HUVECs treated with $VEGF_{121}$/rGel



C: No Treatment Controls

Key Research Accomplishments

- **Described the Pharmacokinetics and Tissue Distribution of $VEGF_{121}$/rGel to allow rational development of an optimal therapeutic schedule on which to base both murine and eventual clinical studies.**
- **Initially described the significant in vivo antitumor effects of $VEGF_{121}$/rGel against orthotopicially-placed breast tumor xenografts. Demonstrated complete regression of primary orthotopic breast tumors in 50% of treated mice(3/6).**
- **Identified significant vascular-ablative effects of $VEGF_{121}$/rGel on breast metastatic foci present in lung. Identified the impact this agent appears to have in supressing the development of tumor metastases by destruction of tumor vascular endotheluim.**
- **Identified a unique aspect of therapy using $VEGF_{121}$/rGel in that lung metastases of treated mice have virtually no vasculature and appear to grow to the approximate limit of oxygen diffusion for avascular tissues.**
- **Identified 20 unique genes asssociated with the development cytotoxic effects of the rGel toxin component on vascular endothelial cells. Confirmed the Gene Chip results via RT-PCR.**
- **Provided significant rationale for continued pre-clinical development of $VEGF_{121}$/rGel as a vascular-ablative agent in breast cancer.**

Reportable Outcomes:

Abstracts:

VEGF121 gelonin fusion protein inhibits breast cancer metastasis in nude mice.Sophia Ran, Khalid Mohamedali, Philip E. Thorpe, Michael G. Rosenblum, Abstract #4 Proceedings of the American Association of Cancer Research 2003.

Complete Manuscripts:

1) Mohamedali, K; Ran, S; Cheung, L; Marks, JW; Waltenberger, J; Thorpe, P and Rosenblum, MG, Mechanistic and Internalization Studies of $VEGF_{121}$/rGel: Cytotoxicity on Endothelial Cells Mediated by VEGFR2 but not by VEGFR1. Molecular Cancer Therapeutics- Submitted for Publication.

2) Ran, S; Mohamedali, K; Thorpe, P; and Rosenblum, MG, The Vascular-Ablative Agent, $VEGF_{121}$/rGel, Inhibits Pulmonary Metastases of MDA-MB-231 Breast Tumors- Journal of the National Cancer Institute- Submitted for Publication.

Conclusions:

Vascular targeting as an approach to tumor therapy holds significant promise for the treatment of solid tumors. However, many current approaches attempting to inhibit the neovascularization process through small molecule inibitors of VEGFR signaling, antibodies to VEGF itself or to the VEGFR2 have not met with success. This is due in part to the multiply-redundant and robust process which tumor vascularization represents. On the other hand, lethal damage to tumor endothelium using the $VEGF_{121}$/rGel fusion toxin is a comparatively unique approach. This construct has remarkable and long-term antitumor effects in xenograft models as opposed to other agents which have limited activity in their own right. Dr. Louise Gorchow , Head of CTEP has indicated in a public presentation that the $VEGF_{121}$/rGel fusion toxin is one of a very few agents with these properties. The data presented above and in the attached Appendix demonstrates that this agent can reduce the growth of both orthotopic breast tumors and can significantly limit the metastatic spread of a breast metastatic model. In addition, the lung metastases which do survive appear to have a much lower vascular supply and a limited tumor cell turnover rate suggesting a reduced growth and metastatic potential. No other vascular targeting agents have thus far demonstrated such unique effects in an in vivo model. Of interest will be to examine the impact this fusion toxin will have on survival in this metastatic model. In addition, our findings examining the mechanism of direct action of the fusion construct on endothelial cells has significance in more exactly understanding how toxins work at the molecular level and may be an important first step in understanding how to more effectively employ these agents for therapeutic advantage. Furthermore, the importance of understanding how vascular targeting agents affect tumor cells indirectly may also have therapeutic significance in understanding the rationale for combinations of these vascular targeting agents with conventional chemotherapeutic agents, or with radiotherapeutic or biological agents.

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chimeric fusion toxin targeting the neovasculature of solid tumors.

Proc.Natl.Acad.Sci.U.S.A, *99*: 7866-7871, 2002.

Appendix:

1 Abstract
2 Manuscripts

Back

Abstract Number: 4

VEGF121 gelonin fusion protein inhibits breast cancer metastasis in nude mice.

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$VEGF_{121}$/rGel fusion protein is a vascular targeting agent composed of a non-heparin binding isoform of VEGF and the highly active plant toxin gelonin. Both receptors for VEGF-A (R1 and R2) are strongly up regulated on tumor neovasculature compared to normal endothelium. We have previously shown that $VEGF_{121}$/rGel was selectively cytotoxic to sub-confluent endothelial cells, displaying an IC_{50} of 0.5 nM compared to 150 nM for free gelonin under the same conditions. Treatment of mice bearing various types of solid tumors with $VEGF_{121}$/rGel (17 mg/kg) inhibited the growth of primary tumors by 65-75% (Veenendaal et al., *PNAS* 99:7866-71, 2002). The goal of this study was to evaluate the effect of $VEGF_{121}$/rGel on metastatic growth of MDA-MB-231 tumor cells in nude mice. The MDA-MB-231 cells (0.5×10^6 per mouse) were injected i.v. and the treatment began 8 days after injection. Mice (6 per group) were treated 6 times either with $VEGF_{121}$/rGel (100 ug/dose) or with an equivalent amount of free gelonin. Three weeks after termination of the treatment, mice were sacrificed and their metastatic lungs as well as all other organs were harvested for examination. The number of surface lung foci in the $VEGF_{121}$/rGel – treated mice was reduced by 58 % as compared to gelonin control animals (mean numbers 22.4 and 53.3 for $VEGF_{121}$/rGel and control, respectively; $p<0.05$). Immunohistochemical analysis revealed that the mean area of lung colonies from $VEGF_{121}$/rGel-treated mice was 50% smaller than that of the control mice (210 ± 37 m m versus 415 ± 10 m m for $VEGF_{121}$/rGel and control, respectively; $p< 0.01$). In addition, the vascularity of metastatic foci from $VEGF_{121}$/rGel-treated mice was reduced compared to that of control colonies. The mean number of blood vessels per mm^2 in metastatic foci was 198 ± 37 versus 388 ± 21 for treated and control, respectively. Approximately 62 % of metastatic colonies from the $VEGF_{121}$/rGel-treated group had fewer than 10 vessels per colony as compared to 23 % in the control group. The VEGF receptor 2, a major receptor that mediates angiogenic effects of VEGF-A, was intensely detected on the metastatic vessels in the control but not on the vessels in the $VEGF_{121}$/rGel-treated group. The treatment was well tolerated. No significant morphological changes were visible in either $VEGF_{121}$/rGel-treated or gelonin control mice. These data strongly suggest that anti-tumor vascular effect of $VEGF_{121}$/rGel could be utilized not only for treating primary tumors but also for inhibiting metastatic spread. This research was funded in part by the Clayton Foundation for Research and DOD Breast Cancer Program.

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Mechanistic and Internalization Studies of $VEGF_{121}$/rGel : Cytotoxicity on Endothelial Cells Mediated by VEGFR2 but not by VEGFR1

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Abstract

We have previously reported the targeting of the neovasculature of solid tumors with $VEGF_{121}$/rGel, a chimeric fusion toxin of $VEGF_{121}$ and recombinant gelonin, a low molecular weight single chain toxin with a mechanism of action similar to that of ricin A-chain. While several studies have shown both receptors of $VEGF_{121}$, namely VEGFR1(FLT-1) and VEGFR2 (KDR/Flk-1), to be over-expressed on the endothelium of tumor vasculature, our *in vitro* studies have shown $VEGF_{121}$/rGel to be cytotoxic to cells over-expressing KDR (IC_{50} = 0.5 nM) but not Flt-1 (IC_{50} = 300 nM), compared to gelonin alone (IC_{50} = 300 nM). Cell ELISA using antibodies specific to either KDR or FLT-1 indicate binding of $VEGF_{121}$/rGel to the both cell lines. Incubation of PAE/KDR and PAE/FLT-1 with ^{125}I-$VEGF_{121}$/rGel demonstrated binding of the fusion toxin to both cell surfaces that was competed with cold $VEGF_{121}$/rGel but not cold gelonin. Both PAE/KDR and PAE/FLT-1 cells were incubated with $VEGF_{121}$/rGel for 1 hr, 4hr, 16 hr and 24 hrs and subjected to immunofluorescence using an anti-rGel antibody. Internalization of $VEGF_{121}$/rGel into PAE/KDR cells within one hour of treatment was observed. However, no $VEGF_{121}$/rGel was detected in PAE/FLT-1 cells up to 24 hours after treatment. Cells treated with rGel only did not immunofluoresce. The cytotoxic mechanism activated as a result of $VEGF_{121}$/rGel internalization is not clear; TUNEL assay over 72 hours did not result in cell death nor was PARP cleavage activated over 48 hours. We conclude that while $VEGF_{121}$/rGel binds to both FLT-1 and KDR, internalization of $VEGF_{121}$/rGel is mediated only by KDR and not FLT-1. $VEGF_{121}$/rGel is also an important molecule useful to probe the biology of KDR and FLT-1 receptors.

Introduction

Angiogenesis is a hallmark of cancer. Vascular endothelial growth factor-A (VEGF-A) plays a key role as the primary stimulant of vascularization in solid tumors[1-3]. VEGF-A enhances endothelial cell proliferation, migration, and survival[3-6] and is essential for blood vessel formation[7]. Other roles of VEGF include wound healing[8], vascular permeability and the regulation of blood flow[9-11]. The VEGF-A family of isomers range from 121 to 206 amino acids in length with varying receptor and heparin binding affinities[25] as a result of alternate splicing[12,13].

VEGF is released by a variety of tumor cells and studies have demonstrated that the VEGF receptor KDR/Flk-1, over-expressed on the endothelium of tumor vasculature, is primarily responsible for mediating the tumor neovascularization properties of VEGF. KDR/Flk-1 is almost undetectable in the vascular endothelium of adjacent normal tissues. $VEGF_{121}$ exists in solution as a disulfide linked homodimer and binds to KDR and FLT-1 in a heparin-independent manner. It does not bind neuropilin-1 or neuropilin-2. $VEGF_{121}$ has been shown to contain the full biological activity of the larger variants.

We have previously defined a novel fusion construct of $VEGF_{121}$ and the highly cytotoxic plant toxin Gelonin (rGel). Gelonin is a 28.5 kDa single-chain toxin with a mechanism of action similar to that of ricin A-chain. The $VEGF_{121}$/rGel fusion toxin was shown to be highly cytotoxic to endothelial cells that over-express the KDR/Flk-1 receptor, but not to cells that over-express the FLT-1 receptor even though $VEGF_{121}$ binds to FLT-1 with greater affinity than to KDR[25]. In this report, we extend our initial findings and further characterize the biological effects of this fusion toxin on endothelial cells. We determined the binding profile of $VEGF_{121}$/rGel to receptors expressed on the cell surface

as well as investigated the internalization of $VEGF_{121}$/rGel.

Materials and Methods

Materials: Bacterial strains, pET bacterial expression plasmids and recombinant enterokinase were obtained from Novagen (Madison, WI). All other chemicals were from Sigma Chemical Company (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA). TALON metal affinity resin was obtained from Clontech laboratories (Palo Alto, CA). Other chromatography resin and materials were from Pharmacia Biotech (Piscataway, NJ). Endothelial cell growth supplement (ECGS) from bovine neural tissue was obtained from Sigma Chemical Company. Murine brain endothelioma (bEnd.3) cells were provided by Professor Werner Risau (Max Plank Institute, Munich, Germany). Tissue culture reagents were from Gibco BRL (Gaithersburg, MD) or Mediatech Cellgro (Herndon, VA). Rabbit anti-gelonin antisera was obtained from the Veterinary Medicine Core Facility at MDACC. Anti-flt-1 (sc-316), anti-flk-1 (sc-504), and anti-PARP (sc-8007) antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Cell culture:. Porcine aortic endothelial cells transfected with the KDR receptor (PAE/KDR) or the FLT-1 receptor (PAE/FLT-1) were a generous gift from Dr. J. Waltenberger. Cells were maintained as a monolayer in F12 Nutrient Media (HAM) suplemented with 100 units/ml penicillin, 100 units/ml streptomycin, and 10% fetal bovine serum and passaged twice weekly. Cells were harvested by treatment with Versene (0.02% EDTA).

Purification of $VEGF_{121}$/rGel: Construction and purification of $VEGF_{121}$/rGel was essentially as described, with minor modifications[26]. E. coli cells were lysed with 100 ml 0.1mm glass beads (BioSpec Products, Inc) in a Bead Beater (BioSped Products, Inc) for

eight cycles of 3 minutes each. The lysate was ultracentrifuged at 40,000 rpm for 90 minutes at 4°C. The supernatant was carefully collected and adjusted to 40 mM Tris-HCl (pH 8.0), 300 mM NaCl, and incubated at 4°C with metal affinity resin. The resin was washed with 40 mM Tris-HCl (pH8.0), 0.5 M NaCl buffer containing 5 mM Imidazole and eluted with buffer containing 100 mM Imidazole. After pooling fractions containing $VEGF_{121}$/rGel, the sample was dialyzed against 20 mM Tris (pH 8.0), 200 mM NaCl and digested with recombinant Enterokinase at room temperature. Enterokinase was removed by agarose-linked soybean trypsin inhibitor. Other proteins of non-interest were removed by Q Sepharose Fast Flow resin and metal affinity resin as described previously[26]. $VEGF_{121}$/rGel was concentrated and stored in sterile PBS at –20°C.

Rabbit Reticulocyte Lysate Assay: The functional activity of rGel and $VEGF_{121}$/rGel were assayed using a cell-free protein translation inhibition assay kit from Amersham Biotech (Arlington Heights, IL) as described by the manufacturer.

ELISA Analysis: The ability of the chimeric fusion protein to bind to Flk-1 was tested on microtiter plates coated with soluble mouse Flk-1. All steps of the ELISA procedure were performed at room temperature unless stated otherwise. Plates were treated with 2 μg/ml of NeutrAvidin (Pierce, Rockford, IL) for 6 h. The extracellular domain of Flk-1 was expressed in Sf9 cells, purified to homogeneity[28], and biotinylated. Biotinylated receptor (1 μg/ml) was incubated with NeutrAvidin-coated wells for 2 h. $VEGF_{121}$ or $VEGF_{121}$/rGel was added to the wells at concentrations ranging from 0.002 to 2 nM in the presence of 2% BSA diluted in PBS or normal mouse serum. After 2 h of incubation, plates were washed

and incubated with non-blocking mouse monoclonal anti-VEGF antibody, 2C3[29] or rabbit polyclonal anti-gelonin IgG. For competition studies of $VEGF_{121}$/rGel and $VEGF_{121}$, binding of the $VEGF_{121}$/rGel fusion protein was detected using a rabbit anti-gelonin antibody. In competition experiments, a 10-fold molar excess of $VEGF_{121}$ was premixed with $VEGF_{121}$/rGel before it was added to the plate. Mouse and rabbit IgG were detected by HRP-labeled goat anti-mouse and anti-rabbit antibodies, respectively (Daco, Carpinteria, CA). Peroxidase activity was measured by adding O-phenylenediamine (0.5 mg/ml) and hydrogen peroxide (0.03% v/v) in citrate-phosphate buffer (pH 5.5). The reaction was stopped by addition of 100 μl of 0.18 M of H_2SO_4. The absorbance was read at 490 nM. Presence of KDR and FLT-1 on cells was tested as follows: 50,000 cells (PAE/KDR or PAE/FLT-1) were aliquoted per well and dried overnight at 37°C. Non-specific binding sites were blocked for 1 hour with 5% BSA. Wells were treated with anti-KDR or anti-FLT-1 antibodies, followed by incubation with HRP-conjugated secondary antibody (1:5000) for 1 hour.

Western Blot Analysis: Whole cell lysates of PAE/KDR and PAE/FLT-1 cells were obtained by lysing cells in Cell Lysis buffer (50 mM Tris, pH 8.0, 0.1 mM EDTA, 1 mM DTT, 12.5 mM $MgCl_2$, 0.1 M KCl, 20% glycerol) supplemented with protease inhibitors (leupeptin (0.5%), aprotinin (0.5%) and PMSF (0.1%)). Protein samples were separated by SDS-PAGE under reducing conditions and electrophoretically transferred to a PVDF memberane overnight at 4°C in transfer buffer (25 mM Tris-HCl, pH 7.6, 190 mM glycine, 20% HPLC-grade methanol). The samples were analyzed for KDR with rabbit anti-flk-1 polyclonal antibody and FLT-1 using an anti-flt-1 polyclonal antibody. The membranes

were then incubated with goat-anti-rabbit IgG horseradish peroxidase (HRP), developed using the Amersham ECL detection system and exposed to X-ray film.

Immunoprecipitation: Cells were lysed as described (see western protocol). 500 μg MDA-MB-231 and L3.6pl cell lysates were mixed with 2 μg anti-flk-1 antibody in a final volume of 250 μl and incubated for two hours at 4°C. 100 μg PAE/KDR and PAE/FLT-1 cell lysates were immunoprecipitated as controls. The mixtures were then incubated overnight with 20 μl Protein A beads that had been blocked with 5% BSA. The beads were washed 4 times in lysis buffer and the samples, along with 30 μg PAE/KDR cell lysate, were run on a gel, transferred overnight onto a PVDF membrane and probed using an anti-flk-1 polyclonal antibody.

Binding of Radiolabeled $VEGF_{121}$/rGel to PAE/KDR and PAE/FLT-1 cells: 100 μg of $VEGF_{121}$/rGel was radiolabeled with 1mCi of NaI^{125} using Chloramine T[27] for a specific activity of 602 Ci/mMol. Cells were grown overnight in 24-well plates. Non-specific binding sites were blocked for 30 minutes with PBS/0.2% gelatin followed by incubation for 4 hours with ^{125}I-$VEGF_{121}$/rGel in PBS/0.2% gelatin solution. For competition experiments, cold $VEGF_{121}$/rGel or gelonin were pre-mixed with ^{125}I-$VEGF_{121}$/rGel. Cells were washed four times with PBS/0.2% gelatin solution, detached and bound cpm was measured.

Cytoxicity of $VEGF_{121}$/rGel and rGel: Cytotoxicity of $VEGF_{121}$/rGel and rGel against log phase PAE/KDR cells was performed as described[26]. Cells were grown in 96 well flat-

bottom tissue culture plates. Purified $VEGF_{121}$/rGel and rGel were diluted in culture media and added to the wells in 5-fold serial dilutions. Cells were incubated for 72 hours. The remaining adherent cells were stained with crystal violet (0.5% in 20% methanol) and solubilized with Sorenson's buffer (0.1 M sodium citrate, pH 4.2 in 50% ethanol). Absorbance was measured at 630 nm. To assess if the activity of $VEGF_{121}$/rGel was affected by the exposure time to endothelial cells, log-phase PAE/KDR cells were grown and treated with $VEGF_{121}$/rGel as above. Media containing the cytotoxic agent was removed at varying time-points and the cells were washed once with 200 μl culture media. Fresh culture media was added to the wells and the cells were then returned to the incubator. Seventy-two hours after the start of the experiment, the number of remaining adherent cells was assessed using crystal violet and Sorensen's buffer as described above.

Internalization of $VEGF_{121}$/rGel into PAE/KDR cells: PAE/KDR and PAE/FLT-1 cells were incubated with 4 μg/ml $VEGF_{121}$/rGel at the timepoints indicated. After stripping the cell surface, cells were fixed with 3.7% formaldehyde and permeabilized with 0.2% Triton X-100. Non-specific binding sites were blocked with 5% BSA in PBS. Cells were then incubated with a rabbit anti-gelonin polyclonal antibody (1:200) followed by a TRITC-conjugated anti-rabbit secondary antibody (1:80). Nuclei were stained with propidium iodide (1μg/ml) in PBS. The slides were fixed with DABCO media, mounted and visualized under fluorescence (Nikon Eclipse TS1000) and confocal (Zeiss LSM 510) microscopes.

TUNEL assay: Log phase PAE/KDR and PAE/FLT-1 cells were diluted to 2000 cells/100 μl. Aliquots (100 μl) were added in 16-well chamber slides (Nalge Nunc International) and incubated overnight at 37°C with 5% CO_2. Purified $VEGF_{121}$/rGel was diluted in culture media and added at 72, 48 and 24 hour time points at a final concentration of 1 nM (twice the IC_{50}). The cells were then processed and analyzed for TUNEL as described by the manufacturer of the reagent. Positive control cells were incubated with 1mg/ml DNAse for 10 minutes at 37°C.

PARP Cleavage: Effects of $VEGF_{121}$/rGel on PARP-mediated apoptosis were investigated by pre-incubating PAE/KDR cells with 100 mM Na_2VO_4 for 5 minutes at 37°C followed by stimulation with $VEGF_{121}$/rGel or $VEGF_{121}$ for 5 minutes, 30 minutes, 4 h, 24 h and 48 h. Cells were washed and lysed and the cell lysate was analyzed by Western using an anti-PARP antibody.

Results

We have previously demonstrated the successful use of $VEGF_{121}$/rGel fusion construct for the targeted destruction of tumor vasculature in vivo. The combination of $VEGF_{121}$ and recombinant gelonin was originally prepared in two different orientations (Figure 1) with both orientations displaying similar cytotoxicity profiles. However, the orientation with $VEGF_{121}$ at the N-terminus results in a higher yield following purification from bacteria (data not shown), and is used in subsequent experiments.

Biological Activity of the rGel Component: The ability of $VEGF_{121}$/rGel and rGel to inhibit translation in a cell-free system was determined by using a rabbit reticulocyte translation assay (Figure 2). The purified $VEGF_{121}$/rGel and rGel had IC_{50} values of 47 and 234 pM, respectively, showing that fusion of rGel and $VEGF_{121}$ did not reduce the activity of the toxin component.

Binding of $VEGF_{121}$/rGel to Soluble Flk-1 Receptor: The fusion protein was tested for its ability to bind to the Flk-1 receptor by ELISA. The extra-cellular domain of recombinant Flk-1 was purified, biotinylated and incubated with wells coated with NeutrAvidin. The receptor was treated with $VEGF_{121}$ or $VEGF_{121}$/rGel, and binding of the ligand to the receptor was assessed by anti-VEGF and anti-gelonin antibodies. Figure 3 shows that $VEGF_{121}$/rGel and native human $VEGF_{121}$ bind equally well to Flk-1 at all concentrations, indicating that the VEGF component of the fusion protein is fully capable of binding to Flk-1. To confirm that the binding of $VEGF_{121}$/rGel to Flk-1 was specific,

free $VEGF_{121}$ (10-fold molar excess) was used to compete with $VEGF_{121}$/rGel for binding to the receptor, followed by detection of $VEGF_{121}$/rGel binding by an anti-gelonin antibody. Binding of $VEGF_{121}$/rGel to the Flk-1 extra-cellular domain was dramatically reduced in the presence of $VEGF_{121}$ (Figure 4) indicating that the $VEGF_{121}$ domain of the fusion toxin retained activity and that binding to the receptor was specific.

$VEGF_{121}$/rGel binds to both KDR and FLT-1: $VEGF_{121}$ has been shown to bind to the FLT-1 receptor with greater affinity than to KDR[25]. Because cytotoxicity of $VEGF_{121}$/rGel to KDR-expressing cells was found to be nearly 600-fold greating than for FLT-1 expressing cells, we investigated the relative binding of $VEGF_{121}$/rGel to PAE cells expressing each of the receptors. ELISA analysis was performed to confirm the expression of both receptors on the cell surface using receptor-specific antibodies (data not shown). Expression of VEGFR-1 (FLT-1) and VEGFR-2 (KDR) was confirmed by western blot (Figure 5a). In order to confirm that $VEGF_{121}$/rGel bound to human VEGFR-1 and VEGFR-2 and that the presence of recombinant gelonin did not interfere with the binding properties of $VEGF_{121}$, we investigated the binding of radiolabeled $VEGF_{121}$/rGel to both KDR and FLT-1 receptors expressed on the surface of PAE cells. Our results (Figure 5b) show that the binding of ^{125}I-$VEGF_{121}$/rGel to both cells was nearly identical. In order to confirm that the binding on the cell surface was receptor-specific, competition studies of ^{125}I-$VEGF_{121}$/rGel with unlabeled $VEGF_{121}$/rGel and rGel were performed. Binding of $VEGF_{121}$/rGel to both PAE/KDR and PAE/FLT-1 cells was competed by unlabeled $VEGF_{121}$/rGel but not by unlabeled gelonin indicating that binding of $VEGF_{121}$/rGel was mediated by $VEGF_{121}$ and, therefore, receptor-specific.

Internalization of $VEGF_{121}$/rGel into PAE/KDR and PAE/FLT-1 cells. We investigated the internalization of $VEGF_{121}$/rGel into PAE/KDR and PAE/FLT-1 cells using immunofluorescence staining. Cells were attached, treated with $VEGF_{121}$/rGel or rGelonin at various time points, and then treated with polyclonal rabbit anti-gelonin primary antibody and FITC-conjugated secondary antibody. $VEGF_{121}$/rGel was detected in PAE/KDR cells within 1 hour of treatment with the immunofluoresence signal progressively increasing to 24 hours (Figure 6). As expected, cell density also decreased over the 24 hour time period. No $VEGF_{121}$/rGel was detected in PAE/FLT-1 cells up to 24 hours after treatment with the fusion toxin. Treatment of cells with the same concentration of rGelonin showed no internalization, confirming that entry of $VEGF_{121}$/rGel into PAE cells was specifically via the KDR receptor.

Exposure duration of $VEGF_{121}$/rGel on endothelial cells: The IC_{50} of $VEGF_{121}$/rGel incubated for 72 hours on log-phase PAE/KDR cells has been shown to be about 1 nM[26]. However, $VEGF_{121}$/rGel internalizes into these cells within one hour of incubation. To study the cytototoxic effect of $VEGF_{121}$/rGel as a function of exposure time of this agent on endothelial cells, we incubated cells with $VEGF_{121}$/rGel from 1-72 hours and assessed its cytotoxicity on PAE/KDR cells at the end of the 72-hour period. While $VEGF_{121}$/rGel retained cytotoxicity even after a one hour incubation, appreciable cytotoxicity was observed after 24 hours and maximal cytotoxic effect of $VEGF_{121}$/rGel on PAE/KDR cells

was observed after 48 hours (Figure 7). The cytotoxic effect of $VEGF_{121}$/rGel on PAE/FLT-1 cells was also affected as a function of exposure duration (data not shown).

TUNEL assay and PARP Cleavage. In order to investigate the mechanism of cytotoxicity of $VEGF_{121}$/rGel to PAE/KDR cells, we performed a TUNEL assay for 24, 48 and 72 hours. No TUNEL staining was observed with PAE/KDR cells exposed to $VEGF_{121}$/rGel up to 72 hours (Figure 8). In contrast nuclei of positive control cells showed intense staining, indicating that the mechanism of cytotoxicity of $VEGF_{121}$/rGel is not apoptotic. PARP cleavage was tested on PAE/KDR cells by treating cells with $VEGF_{121}$/rGel or $VEGF_{121}$ for periods ranging from 5 minutes to 48 hours. Western blot analysis of these cells by an anti-PARP antibody shows that $VEGF_{121}$/rGel did not activate PARP-mediated apoptosis (Figure 9).

Discussion

Agents targeting the neovascularization process in tumors have significant potential for therapeutic impact. Molecules which interfere with the growth and development of vascular endothelial cells by targeting the VEGF/receptor complex have an additional advantage since these agents do not have to penetrate into the tumor parenchyma and the receptor targets are expressed on the luminal surface of tumor vascular endothelium.

Although this study demonstrates that the VEGF/rGel fusion can bind to both the KDR and FLT-1 receptors, we found that only cells which express KDR were able to internalize the construct thereby delivering the toxin component to the cytoplasmic compartment. Zheng et al [3] suggest that it is the interaction of VEGF with the KDR receptor but not the FLT-1 receptor which is responsible for the growth proliferative signal on endothelial cells and other studies suggest that the KDR receptor is primarily responsible for mediating the vascular permeability effects of VEGF-A [14]. Studies suggest that the FLT-1 receptor can modulate signaling of the KDR receptor[3] and may impact monocyte response to VEGF[15], but its role in neovascularization has not been well-defined.

The possible binding of VEGF-containing constructs to the neuropilin receptor could be a source of unwanted toxicity and mis-targeting of the complex, however, studies by Gluzman et al.[16] have demonstrated that the $VEGF_{121}$ fragment as opposed to other forms of VEGF-A does not appear to bind to this receptor.

Another important aspect of this study was the observation that the cytotoxic effects of the construct on vascular endothelial cells did not involve an apoptotic mechanism. This is in sharp contrast to studies of other toxins such as ricin A chain (RTA)

and pseudomonas exotoxin (PE) which demonstrate generation of apoptotic effects which appear to be mediated, at least in part, by caspase activation[17-19]. Recently, Keppler-Hafkemeyer, et al[20] have suggested that PE toxins may generate cytotoxic effects through both caspase-dependant and protein synthesis inhibitory mechanisms. Despite the sequence homology of RTA and rGel[21] and the known similarities in their mechanism of action[22,23], it appears that these two toxins differ in their pro-apoptotic effects. One possible explanation for the observed differences in apoptotic effects between RTA and rGel toxin could be in the cell types examined. The cells targeted in the current study of rGel are non-transformed endothelial cells while those in the RTA study were tumor cells.

The exposure duration studies for the $VEGF_{121}$/rGel fusion toxin demonstrate that as little as 1 hr exposure to target cells is required to develop a cytotoxic effect 72 hrs later. However, continual exposure for up to 48 hrs was shown to improve the cytotoxic effect by almost 10 fold. Should pharmacokinetic studies demonstrate a relatively short plasma half-life for this agent, this may suggest that optimal therapeutic effect could be achieved by maintaining blood concentrations of drug at therapeutic concentrations for at least 48 hrs. This could be achieved by frequent interval dosing or continuous infusion but may be important in the development of pre-clinical and clinical dosing strategies.

Figure Legends

Figure 1: Design and construction of $VEGF_{121}$/rGel. Constructs of the targeting molecule ($VEGF_{121}$) to the cytotoxic agent (gelonin) were expressed in two orientations, with either $VEGF_{121}$ or gelonin at the N-terminus. A G4S tether was used to fuse $VEGF_{121}$ and gelonin and reduce steric hindrance.

Figure 2: Rabbit reticulocyte assay to determine the ability of $VEGF_{121}$/rGel and rGel to inhibit translation in a cell-free system. The fusion of $VEGF_{121}$ and recombinant gelonin does not reduce the activity of the toxin component.

Figure 3: ELISA demonstrating that $VEGF_{121}$/rGel binds to the receptor. $VEGF_{121}$/rGel, $VEGF_{121}$ and rGel were incubated with biotinylated mouse flk-1 receptor attached to NeutrAvidin-coated plates. Binding was assessed using anti-gelonin and anti-VEGF antibodies.

Figure 4: Binding to the anchored flk-1 receptor is specific for $VEGF_{121}$/rGel. $VEGF_{121}$/rGel or $VEGF_{121}$ was incubated with flk-1 receptor as described in Materials and Methods. Binding of $VEGF_{121}$/rGel was competed with $VEGF_{121}$ and a rabbit anti-gelonin antibody was used for detection. $VEGF_{121}$ specifically reduced binding of $VEGF_{121}$/rGel to flk-1. $VEGF_{121}$ was not detected by the anti-gelonin antibody (data not shown).

Figure 5: Expression of KDR and FLT-1. (A) Whole cell lysate (30 μg) of PAE/KDR and PAE/FLT-1 was run on an SDS-PAGE gel, transferred to a PVDF membrane and immunoblotted using the appropriate antibody. Expression of both receptors on their respective cell-lines was confirmed. (B) Receptor-specific binding of radio-labeled $VEGF_{121}$/rGel is demonstrated on cells expressing these receptors. Binding was reduced with unlabeled $VEGF_{121}$/rGel but not by unlabeled gelonin.

Figure 6: Internalization of $VEGF_{121}$/rGel into PAE/KDR and PAE/FLT-1 cells. PAE/KDR cells were incubated with 4 μg/ml $VEGF_{121}$/rGel at the timepoints indicated. Cells were then incubated with an anti-gelonin polyclonal antibody (1:200) followed by a FITC-conjugated secondary antibody (1:80). Nuclei were stained with propidium iodide. $VEGF_{121}$/rGel enters PAE/KDR cells within one hour of treatment. However, PAE/FLT-1 cells did not internalize $VEGF_{121}$/rGel even after 24 hours of incubation with $VEGF_{121}$/rGel.

Figure 7: Effect of exposure time of $VEGF_{121}$/rGel on PAE/KDR cells on cytotoxicity. $VEGF_{121}$/rGel was incubated with PAE/KDR cells for varying lengths of time, as described in Materials and Methods. While $VEGF_{121}$/rGel retained cytotoxicity towards PAE/KDR cells even with a 1 h exposure time, cytotoxicity of this fusion toxin was markedly enhanced by an exposure time of 48 hours.

Figure 8: Cytotoxicity of $VEGF_{121}$/rGel to PAE/KDR cells does not result in apoptosis. PAE/KDR cells were grown overnight. 1 nM $VEGF_{121}$/rGel (twice the IC_{50})

was added and incubated for 24, 48 and 72 hours. The cells were analyzed for TUNEL. Positive control cells were incubated with 1 mg/ml DNAse for 10 minutes at 37°C.

Figure 9: Treatment of PAE/KDR cells with $VEGF_{121}$/rGel does not result in PARP cleavage. PAE/KDR cells were stimulated with $VEGF_{121}$/rGel or $VEGF_{121}$ for the times indicated. Cells were washed and lysed and the cell lysate was analyzed by Western using an anti-PARP antibody. No PARP cleavage was observed.

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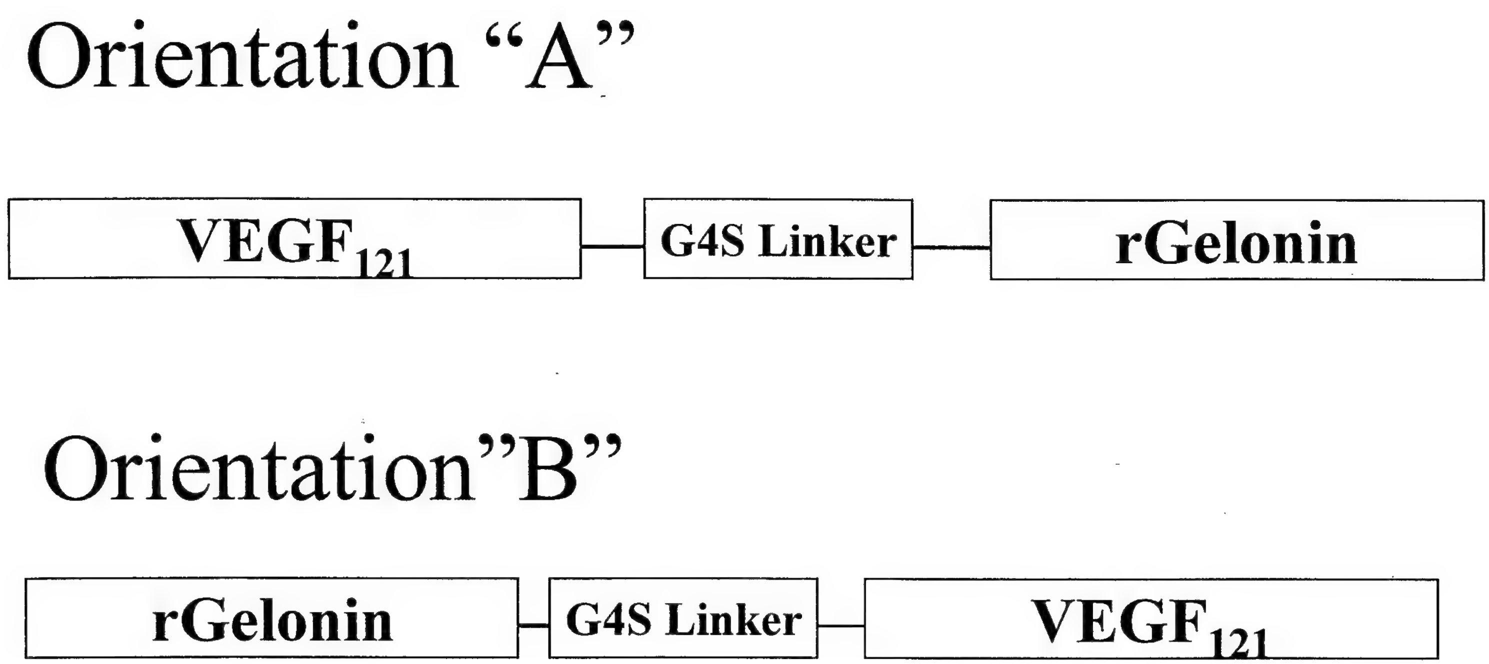
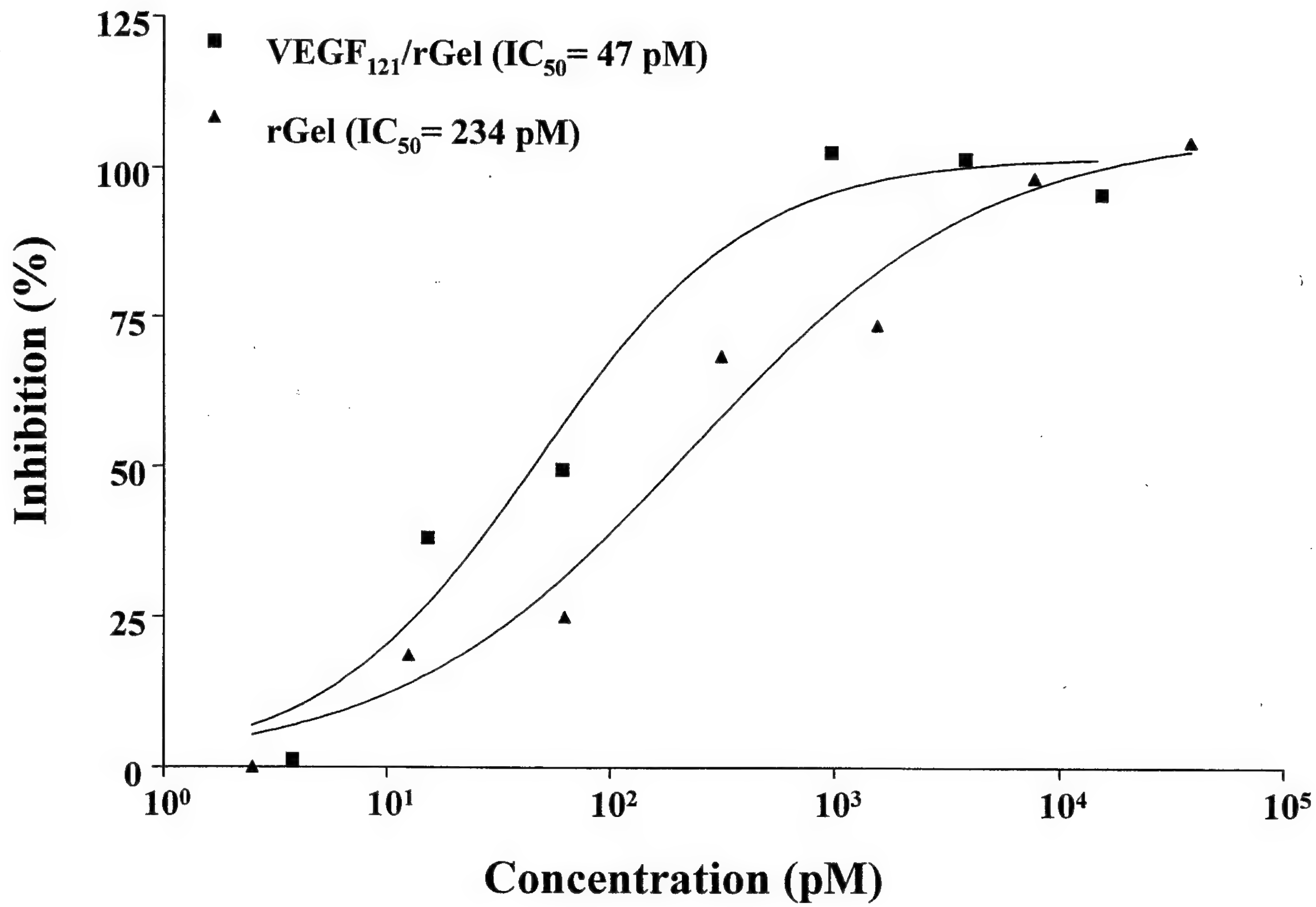
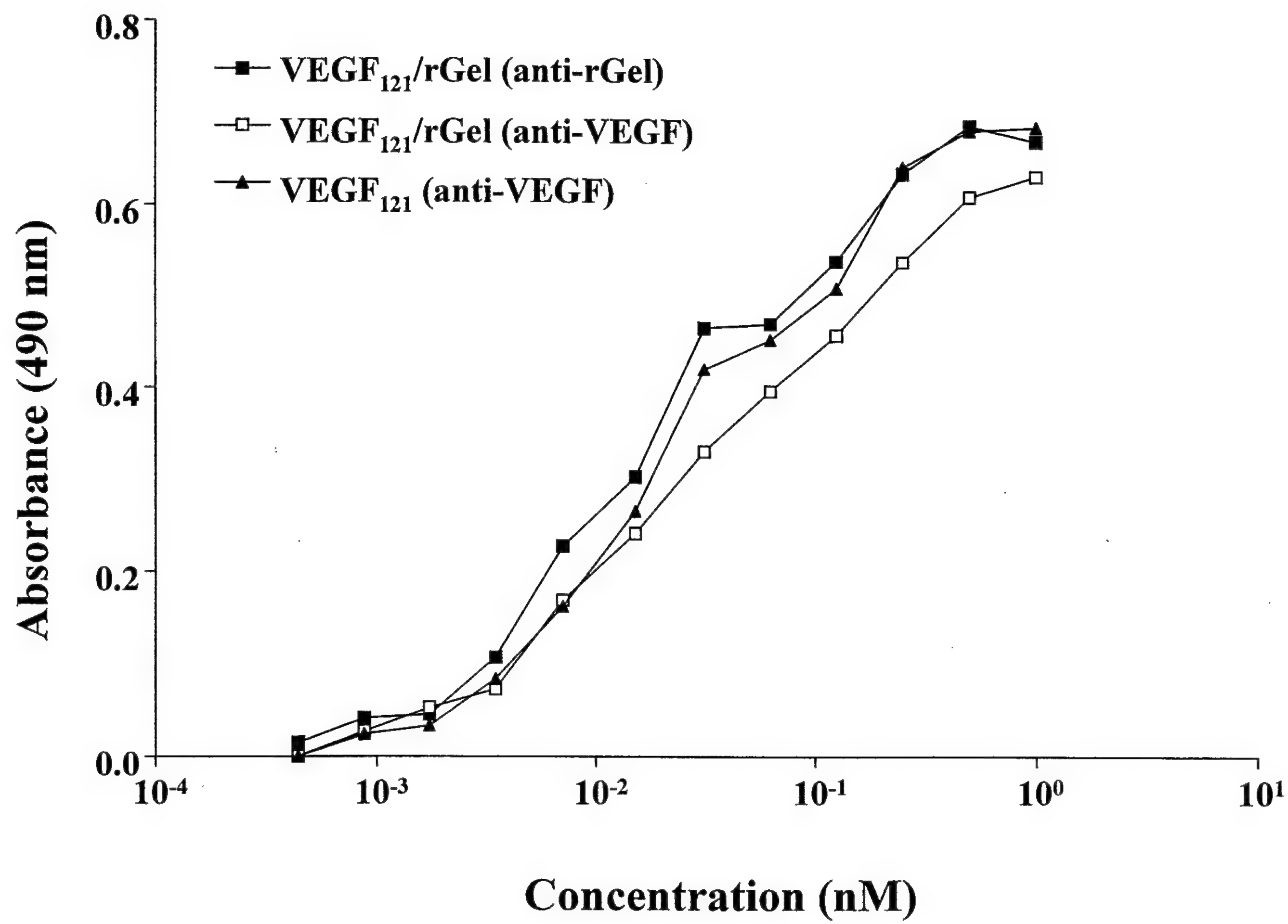

Orientation “A”
VEGF121
G4S Linker
rGelonin
Orientation”B”
rGelonin
G4S Linker
VEGF121

Figure 1



VEGF121/rGel (IC50= 47 pM)
rGel (IC50= 234 pM)
Inhibition (%)
125
100
75
50
25
0
10^0
10^1
10^2
10^3
10^4
10^5
Concentration (pM)

Figure 2



0.8
0.6
0.4
0.2
0.0
Absorbance (490 nm)
VEGF121/rGel (anti-rGel)
VEGF121/rGel (anti-VEGF)
VEGF121 (anti-VEGF)
10-4
10-3
10-2
10-1
100
101
Concentration (nM)

Figure 3

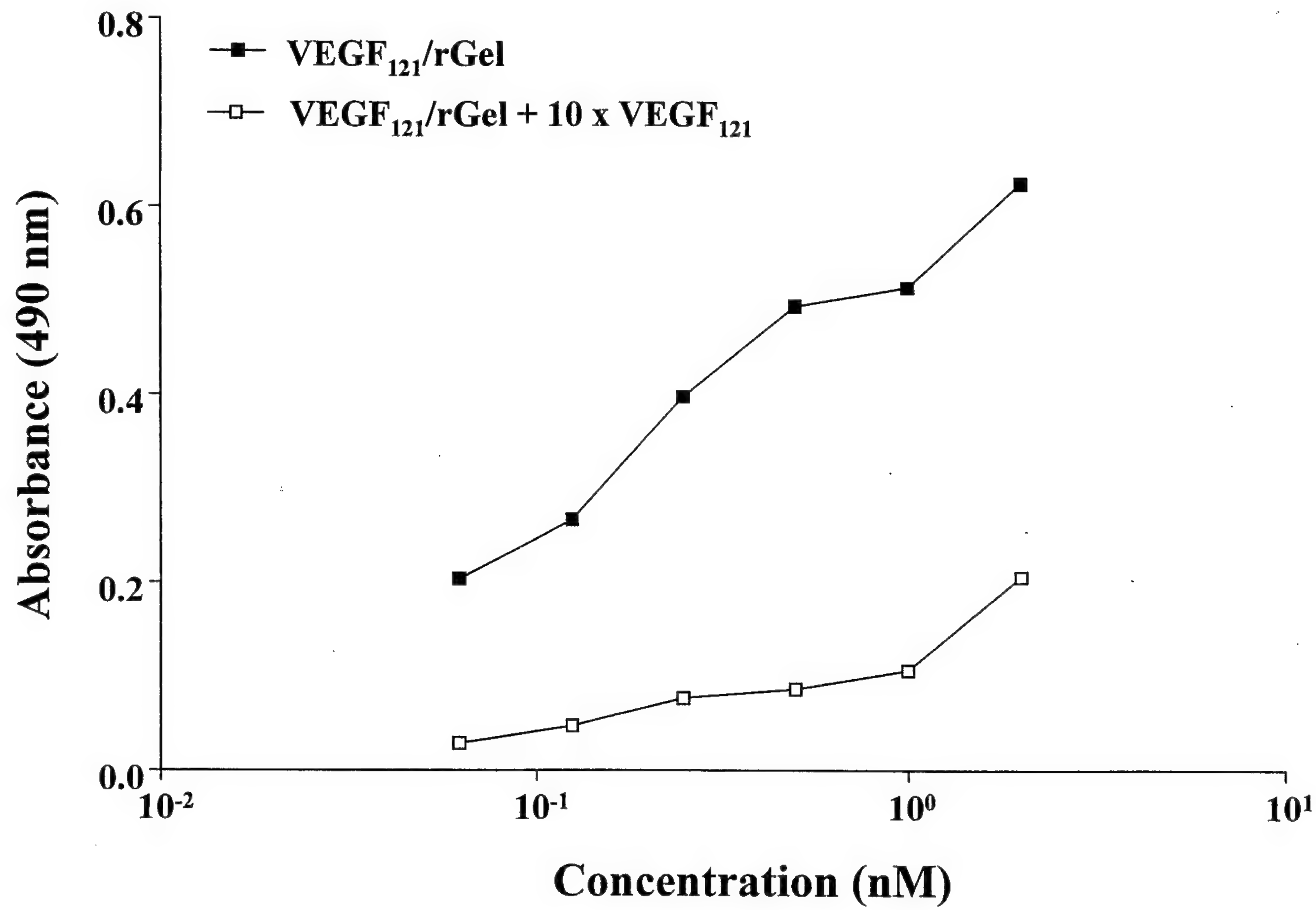
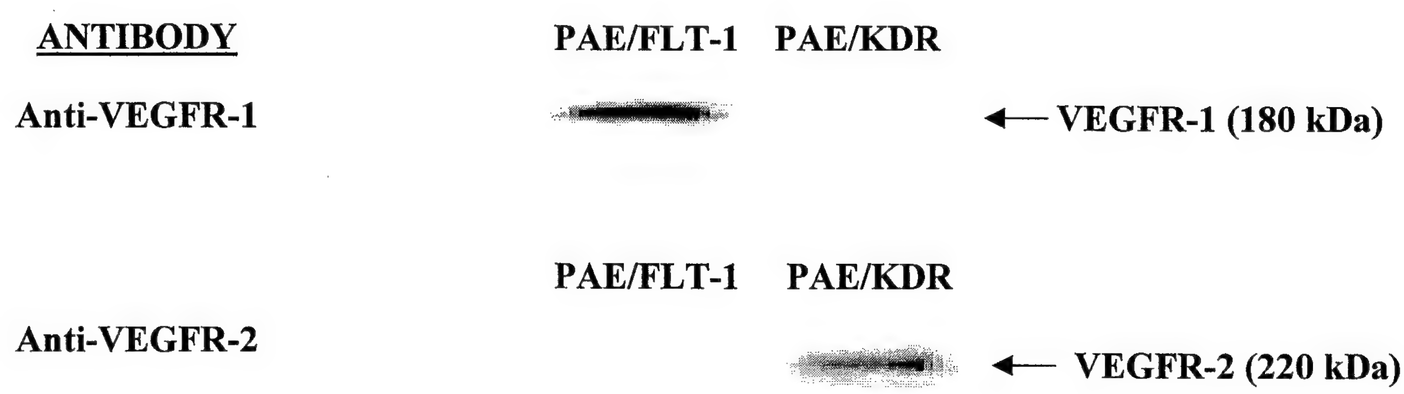

VEGF121/rGel
VEGF121/rGel + 10 x VEGF121
Absorbance (490 nm)
0.8
0.6
0.4
0.2
0.0
10-2
10-1
100
101
Concentration (nM)

Figure 4

Western Analysis of VEGF Receptors



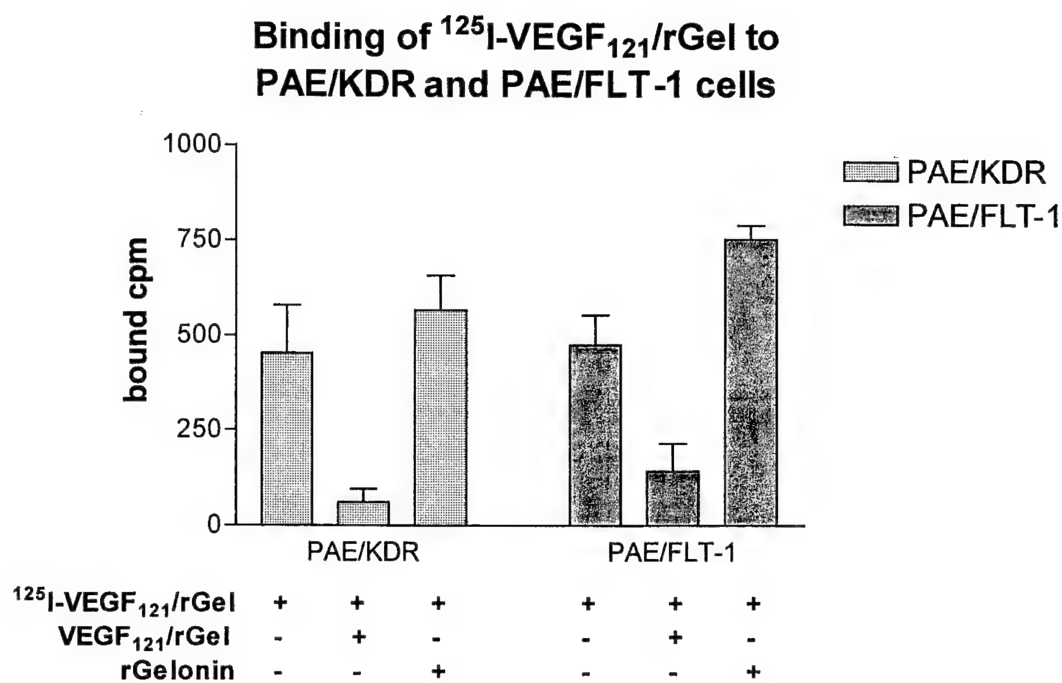


Figure 5

Internalization of $VEGF_{121}$/rGel into PAE/KDR and PAE/FLT-1 cells

PAE/FLT-1

1 h

4 h

24 h

PAE/KDR

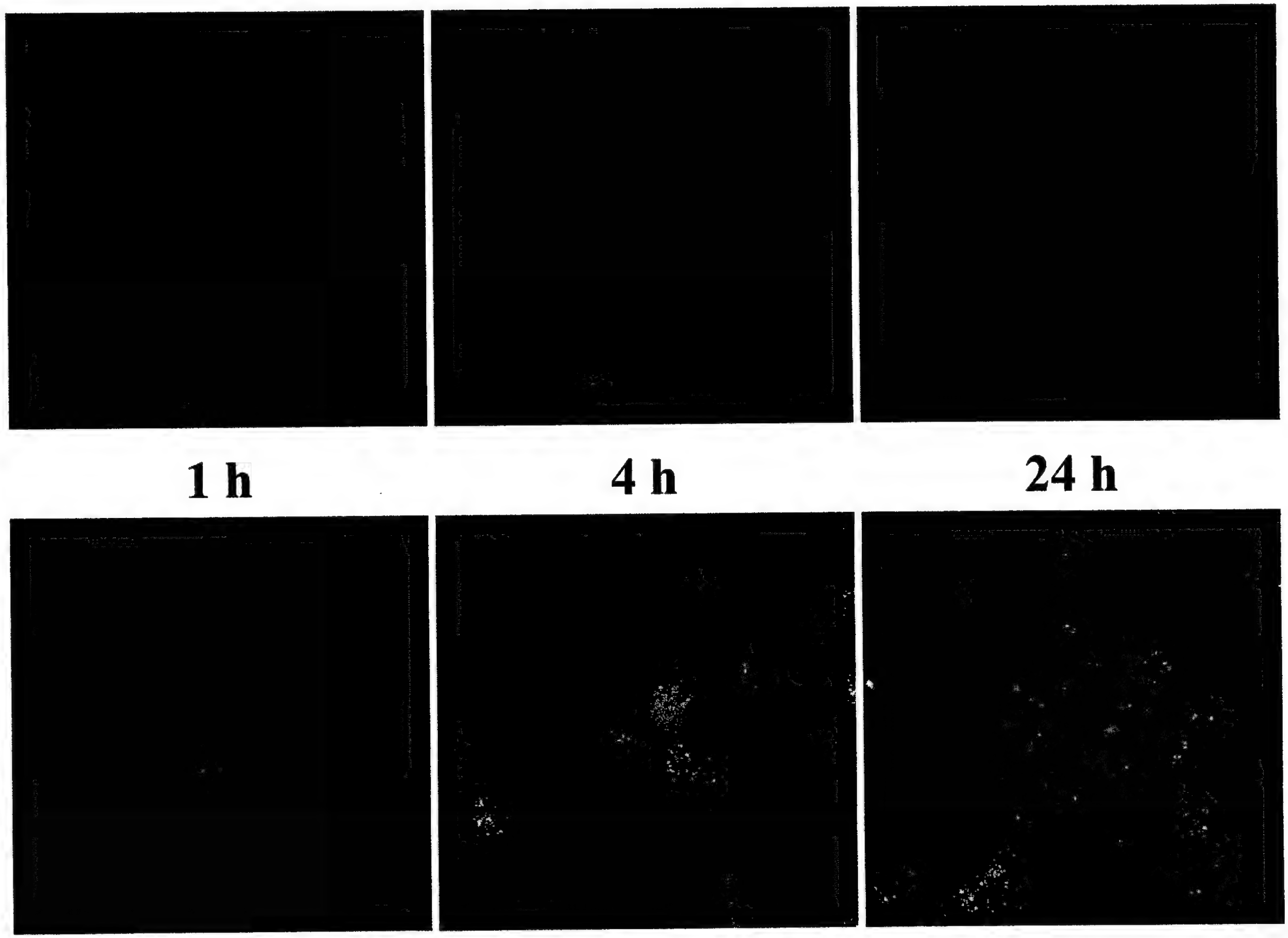
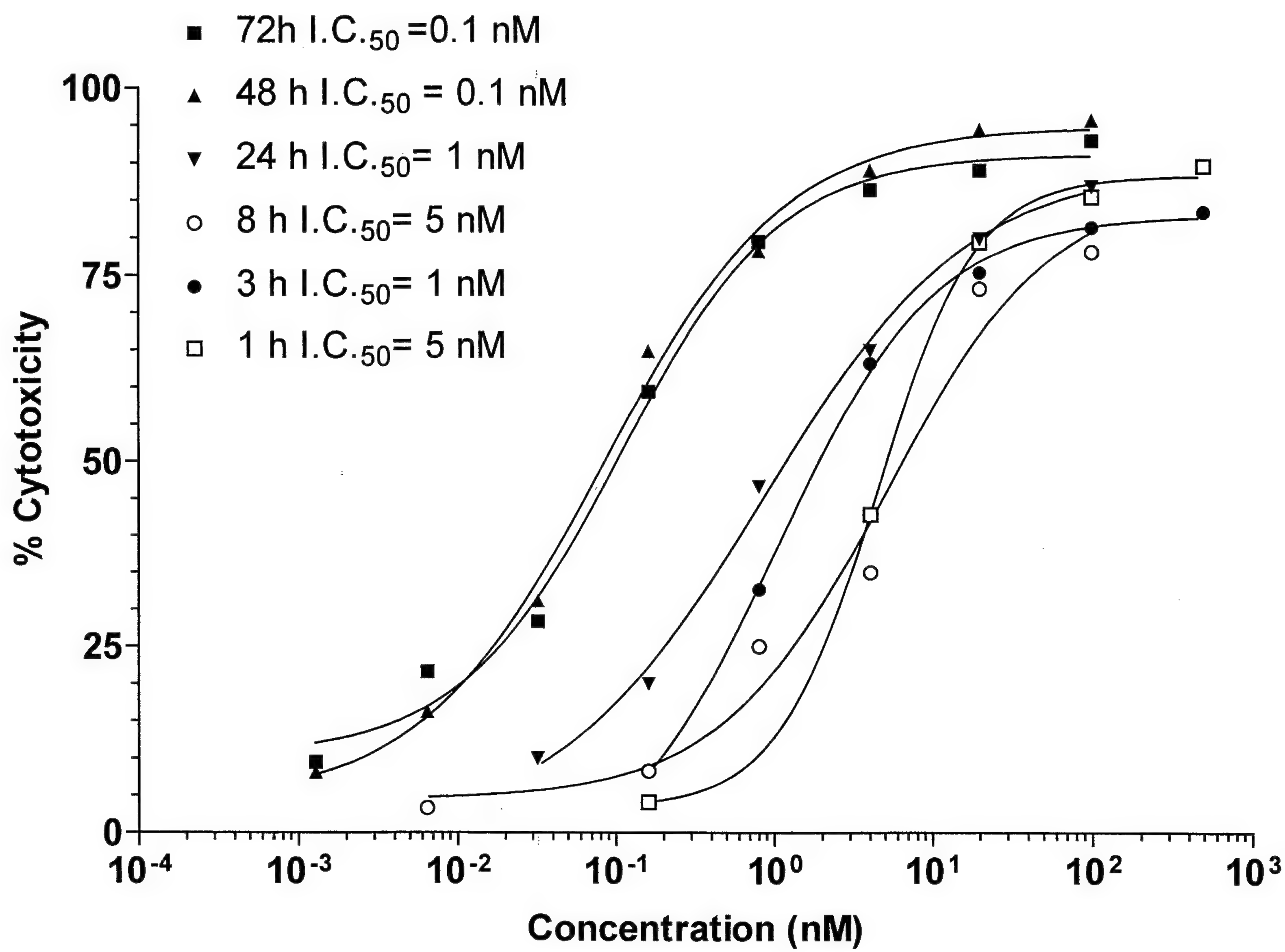


Figure 6



72h I.C.50 =0.1 nM
48 h I.C.50 = 0.1 nM
24 h I.C.50= 1 nM
8 h I.C.50= 5 nM
3 h I.C.50= 1 nM
1 h I.C.50= 5 nM
% Cytotoxicity
100
75
50
25
0
10-4
10-3
10-2
10-1
100
101
102
103
Concentration (nM)

Figure 7

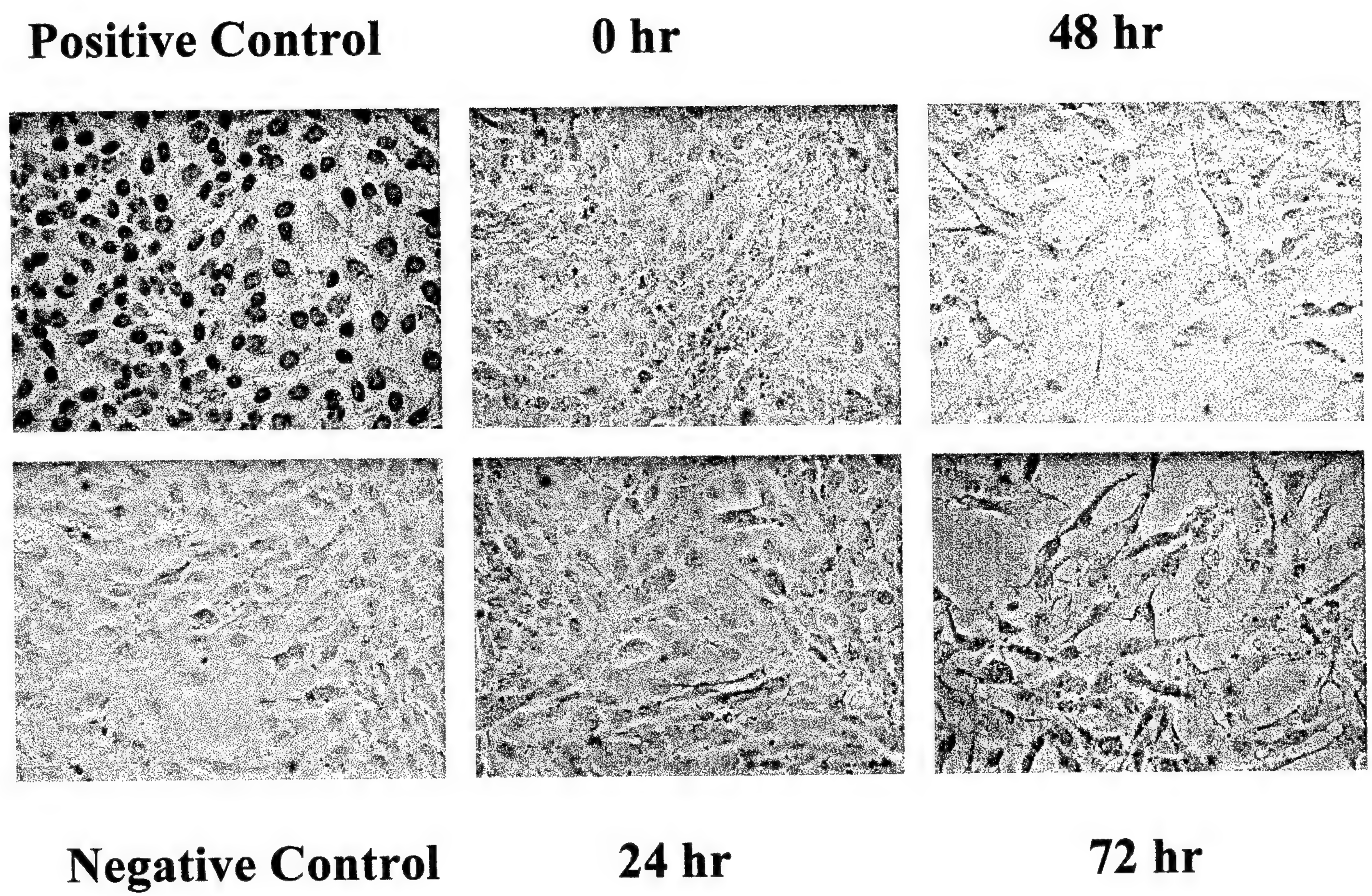


Figure 8

The Vascular-Ablative Agent, $VEGF_{121}$/rGel, Inhibits Pulmonary Metastases of MDA-MB-231 Breast Tumors

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Abbreviations: $VEGF_{121}$/rGel, vascular endothelial growth factor 121 fused with recombinant toxin gelonin; VEGFR2; VEGF receptor 2; HRP, horseradish peroxidase; HPF, high power field.

Key Words: breast cancer; lung metastases; inhibition; $VEGF_{121}$/rGel fusion toxin; vascular targeting agent

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ABSTRACT

We evaluated the effect of $VEGF_{121}$/rGel fusion toxin treatment on the growth of metastatic MDA-MB-231 tumor cells in nude mice. Tumor cells ($0.5x10^6$ per mouse) were injected i.v. and 8 days after innoculation, mice (6 per group) were treated 6 times either with $VEGF_{121}$/rGel (100 ug/dose) or free gelonin. Three weeks after treatment, mice were sacrificed and the lungs were harvested and examined. The surface lung foci in the $VEGF_{121}$/rGel – treated mice were reduced by 58 % as compared to gelonin control animals (means were 22.4 and 53.3 for $VEGF_{121}$/rGel and control, respectively; $p<0.05$). The mean area of lung colonies from $VEGF_{121}$/rGel-treated mice was also 50% smaller than control mice (210 ± 37 μm versus 415 ± 10 μm for $VEGF_{121}$/rGel and control, respectively; $p< 0.01$). In addition, the vascularity of metastatic foci as assessed by the mean number of blood vessels per mm^2 in metastatic foci was significantly reduced (198 ± 37 versus 388 ± 21 for treated and control, respectively). Approximately 62% of metastatic colonies from the $VEGF_{121}$/rGel-treated group had fewer than 10 vessels per colony as compared to 23% in the control group. The VEGF receptor(flk-1) was intensely detected on the metastatic vessels in the control but not on the vessels in the $VEGF_{121}$/rGel-treated group. Metastatic foci present in lung had a 3-fold lower Ki-67 labeling number compared to control tumors. These data strongly suggest that the anti-tumor vascular-ablative effect of $VEGF_{121}$/rGel could be utilized not only for treating primary tumors but also for inhibiting metastatic spread.

INTRODUCTION

Biological studies examining the development of the vascular tree in normal development and in disease states have identified numerous cytokines and their receptors responsible for triggering and maintaining this process *(1-7)*. Tumor neovascularization is central not only to the growth and development of the primary lesion but appears to be a critical factor in the development and maintenance of metastases *(8-12)*. Clinical studies in bladder cancer *(9)* have demonstrated a correlation between micro-vessel density and metastases. In addition, studies of breast cancer metastases by Fox et al. and Aranda et al. *(11-12)* have demonstrated that microvessel count in primary tumors appears to be related to the presence of metastases in lymph nodes and micrometastases in bone marrow.

The cytokine vascular endothelial growth factor-A (VEGF-A) and its receptors Flt-1 and KDR have been implicated as one of the central mediators of normal angiogenesis and tumor neovascularization *(13-20)*. Up-regulation or over-expression of the KDR receptors or the VEGF-A ligand itself has been implicated as poor prognostic markers in various clinical studies of colon, breast and pituitary cancers *(21-23)*. Recently, Padro et al. *(24)* have suggested that both VEGF-A and KDR may play a role in the neovascularization observed in bone marrow during AML tumor progression and may provide evidence that the VEGF/KDR pathway is important in leukemic growth.

For these reasons, there have been several groups interested in developing therapeutic agents and approaches targeting the VEGF-A pathway. Agents which prevent VEGF-A binding to its receptor, antibodies which directly block the KDR receptor itself and small molecules which

block the kinase activity of the KDR and thereby block growth factor signaling are all under development *(25-37)*. Recently, our laboratory reported the development of a growth factor fusion construct of $VEGF_{121}$ and the recombinant toxin gelonin *(38)*. Our studies demonstrated that this agent was specifically cytotoxic only to cells expressing the KDR receptor and was not cytotoxic to cells over-expressing the Flt-1 receptor. In addition, this agent was shown to localize within tumor vasculature and caused a significant damage to vascular endothelium in both PC-3 prostate and MDA-MB-231 orthotopic xenograft tumor models.

The current study seeks to extend our original observations describing the in vitro biological effects of this novel fusion construct and we examined the effects of this agent in both orthotopic and metastatic tumor models.

MATERIALS AND METHODS

Materials

Bacterial strains, pET bacterial expression plasmids and recombinant enterokinase were obtained from Novagen (Madison, WI). All other chemicals were from Sigma Chemical Company (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA). TALON metal affinity resin was obtained from Clontech laboratories (Palo Alto, CA). Other chromatography resin and materials were from Pharmacia Biotech (Piscataway, NJ). Endothelial cell growth supplement (ECGS) from bovine neural tissue was obtained from Sigma Chemical Company. Murine brain endothelioma (bEnd.3) cells were provided by Professor Werner Risau (Max Plank Institute, Munich, Germany). Tissue culture reagents were from Gibco BRL (Gaithersburg, MD) or Mediatech Cellgro (Herndon, VA).

Antibodies

Rat anti-mouse CD31 antibody was from PharMingen (San Diego, CA). Rabbit anti-gelonin antibody was produced in the Veterinary Medicine Core Facility at MDACC. Hybridoma producing the mouse monoclonal 6w/32 antibody directed against human HLA antigen was purchased from ATCC ( ). The 6w/32 antibody was purified from hybridoma supernatant using Protein A resin. MECA 32, a pan mouse endothelial cell antibody, was kindly provided by Dr. E. Butcher (Stanford University, CA) and served as a positive control for immunohistochemical studies. The ki-67 antibody was from Abcam, Inc(Cambridge, UK). Goat anti-rat, anti-mouse and anti-rabbit secondary antibodies conjugated to HRP were purchased from Daco (Carpinteria, CA).

Methods

Cell Culture

Porcine aortic endothelial cells transfected with the KDR receptor (PAE/KDR) or the Flt-1 receptor (PAE/Flt-1) were a generous gift from Dr. J. Waltenberger. MDA-MB-231 cells were a generous gift from Dr. Janet Price. Cells were maintained as a monolayer in F12 Nutrient Media (HAM) supplemented with 100 units/ml penicillin, 100 units/ml streptomycin, and 10% fetal bovine serum. Cells were harvested by treatment with Versene (0.02%EDTA). Tumor cells intended for injection into mice were washed once and resuspended in serum-free medium without supplements. Cell number and viability were determined by staining with 0.2% trypan blue dye diluted in saline. Only single-cell suspensions of greater than 90% viability were used for *in vivo* studies.

Expression and Purification of $VEGF_{121}$/rGel

The construction, expression and purification of $VEGF_{121}$/rGel has been previously described *(38)*. The fusion toxin was stored in sterile PBS at -20°C.

Cytoxicity of $VEGF_{121}$/rGel and rGel

Cytotoxicity of $VEGF_{121}$/rGel and rGel against log phase PAE/KDR and PAE/Flt-1 cells have been previously described *(38)*. Here, we assessed the cytotoxicity of $VEGF_{121}$/rGel and rGel against log phase MDA-MB-231 cells. Cells were grown in 96 well flat-bottom tissue culture plates. Purified $VEGF_{121}$/rGel and rGel were diluted in culture media and added to the wells in 5-fold serial dilutions. Cells were incubated for 72 hours. The remaining adherent cells were stained with crystal violet (0.5% in 20% methanol) and solubilized with Sorenson's buffer (0.1 M sodium citrate, pH 4.2 in 50% ethanol). Absorbance was measured at 630 nm.

Western Blot Analysis

Whole cell lysates of PAE/KDR and PAE/Flt-1 cells were obtained by lysing cells in Cell Lysis buffer (50 mM Tris, pH 8.0, 0.1 mM EDTA, 1 mM DTT, 12.5 mM $MgCl_2$, 0.1 M KCl, 20% glycerol) supplemented with protease inhibitors (leupeptin (0.5%), aprotinin (0.5%) and PMSF (0.1%). Protein samples were separated by SDS-PAGE under reducing conditions and electrophoretically transferred to a PVDF memberane overnight at 4°C in transfer buffer (25 mM Tris-HCl, pH 7.6, 190 mM glycine, 20% HPLC-grade methanol). The samples were analyzed for KDR with rabbit anti-flk-1 polyclonal antibody (Santa Cruz Biotechnology, Inc.) and Flt-1 using an anti-flt-1 polyclonal antibody (Santa Cruz Biotechnology, Inc.). The membranes were then

incubated with goat-anti-rabbit IgG horseradish peroxidase (HRP), developed using the Amersham ECL detection system and exposed to X-ray film.

Immunoprecipitation

Cells were lysed as described (see western protocol). 500 μg MDA-MB-231 cell lysate was mixed with 2 μg anti-flk-1 antibody in a final volume of 250 μl and incubated for two hours at 4°C. 100 μg PAE/KDR and PAE/Flt-1 cell lysates were immunoprecipitated as controls. The mixtures were then incubated overnight with 20 μl Protein A beads that had been blocked with 5% BSA. The beads were washed 4 times in lysis buffer and the samples, along with 30 μg PAE/KDR cell lysate, were run on a gel, transferred overnight onto a PVDF membrane and probed using an anti-flk-1 polyclonal antibody.

Localization of $VEGF_{121}$/rGel to Blood Vessels of MDA-MB-231 Tumors

SCID mice (3 mice per group) bearing MDA-MB-231 tumors were intravenously injected with 50 ug of the fusion protein or equivalent amount of free gelonin. The mean tumor volume per group was 260 mm^3. Thirty minutes later mice were sacrificed and exsanguinated. All major organs and tumor were harvested and snap-frozen for preparation of cryosections. Frozen sections were double stained with a pan-endothelial marker MECA 32 (5 ug/ml) followed by detection of the localized fusion protein using rabbit anti-gelonin antibody (10 ug/ml). MECA 32 rat IgG was visualized by goat anti-rat IgG conjugated to FITC (green fluorescence). Rabbit anti-gelonin antibody was detected by goat anti-rabbit IgG conjugated to Cy-3 (red fluorescence). Co-localization of both markers was indicated by yellow color. Anti-gelonin antibody had no reactivity with tissues sections derived from mice injected with saline or with $VEGF_{121}$. To

determine % of vessels with localized fusion protein, MECA 32 positive, gelonin-positive and vessels with combined color were counted at magnification of x 200 in at least 10 fields per section. Two slides from each mouse were analyzed and percent of positive vessels was averaged.

Metastatic Model of MDA-MB-231 Tumors

Female SCID mice, aged 4-5 weeks, were injected in a tail vein with 0.1 ml of MDA-MB-231 cell suspension ($5x10^5$ cells). The mice were randomly separated into two groups (6 mice per group) and were treated with either $VEGF_{121}$/rGel or gelonin alone (100 μg intraperitoneally, 6 times total with the interval of 3 days) starting the 8^{th} day after the injection of cells. Three weeks after termination of the treatment, the animals were sacrificed and their lungs were removed. One lobe was fixed in Bouin's fixative and the other lobe was snap-frozen. After fixation in Bouin's fixative, the tumor colonies on the lung surface appear white, whereas the normal lung tissue appears brown. The number of tumor colonies on the surface of each lung was counted and the weight of each lung was measured. The values obtained from individual mice in the $VEGF_{121}$/rGel and rGel groups were averaged per group.

Determination of Number, Size and Vascular Density of Lung Metastatic Foci

Frozen samples of lung tissue was cut to produce sections of 6 μm. Blood vessels were visualized by MECA 32 antibody and metastatic lesions were identified by morphology and by 6w/32 antibody, directed against human HLA antigens. Each section was also double stained by MECA 32 and 6w/32 antibodies to ensure that the analyzed blood vessels are located within a metastatic lesion. Slides were first viewed at low magnification (x 2 objective) to determine total

number of foci per a cross-section. Six slides derived from individual mice in each group were analyzed and the number was averaged. Images of each colony were taken using digital camera (CoolSnap) at magnifications of x40 and x100 and analyzed using Metaview software that allows measurements of smallest and largest diameter, perimeter (μm) and area (mm^2). The vascular endothelial structures identified within a lesion were counted and number of vessels per each lesion was determined and normalized per mm^2. The mean number of vessels per mm^2 was calculated per each slide and averaged per $VEGF_{121}$/rGel and rGel groups (6 slides per group). The results are expressed ± SEM. The same method applied to determine the mean number of vessels in non-malignant tissues.

Immunohistochemical Analysis of Proliferation of Tumor Cells in the Lung Colonies

Frozen sections of mouse normal organs and metastatic lungs were fixed with acetone for 5 min and rehydrated with PBST for 10 min. All dilutions of antibodies were prepared in PBST containing 0.2% BSA. Primary antibodies were detected by appropriate anti-mouse, anti-rat or anti-rabbit HRP conjugates. HRP activity was detected by developing with DAB substrate (Research Genetics). To determine number of cycling cells sections were stained with the ki-67 antibody followed by anti-mouse IgG HRP conjugate. Sections were analyzed at magnification of x100. Number of cells positive for ki-67 was normalized per mm^2. The mean number ± SD per $VEGF_{121}$/rGel and control group is presented. The average numbers derived from analysis of each slide were combined per either $VEGF_{121}$/rGel or rGel group and analyzed for statistical differences.

Expression of KDR in metastatic Lung Tumors

The expression of VEGF receptor-2 on the vasculature of breast tumors metastatic to lung was also assessed using the RAF-1 antibody as described in(**Evaluation of novel anti-mouse VEGFR2 antibodies as potential anti-angiogenic or vascular targeting agents for tumor therapy.** Sophia Ran, Xianming Huang, Amber Downes and Philip E. Thorpe- Neoplasia in press)

Frozen sections of lungs from mice treated with $VEGF_{121}$/rGel or free gelonin stained with monoclonal rat anti-mouse VEGFR2 antibody RAFL-1 (10 μg/ml). RAFL-1 antibody was detected by goat anti-rat IgG-HRP

Statistical Analysis

Results are expressed as mean ± SEM unless otherwise indicated. Statistical significance was determined by one way analysis of variance followed by the Student's t-test.

Results

Cytotoxicity of $VEGF_{121}$/rGel on MDA-MB-231 Cells

We have previously demonstrated that $VEGF_{121}$/rGel is cytotoxic to endothelial cells expressing KDR but not Flt-1. As assessed by Western, MDA-MB-231 cells do not appear to express VEGFR1 or VEGFR2, the receptors which bind $VEGF_{121}$. We additionally examined the cytotoxicity of $VEGF_{121}$/rGel on MDA-MB-231cells in culture and showed an IC_{50} slightly higher than that observed for recombinant gelonin (Fig. 1), indicating that $VEGF_{121}$/rGel does not have a specific target on MBA-MB-231 cells.

Localization of $VEGF_{121}$/rGel to Vasculature of MDA-MB-231 Tumors

Mice bearing orthotopic MDA-MB-231 tumors were injected intravenously with either $VEGF_{121}$/rGel (50 ug/mouse) or free gelonin (20 ug/mouse) and, thirty minutes later, the mice were exsanguinated. Frozen sections were prepared from the tumor and normal organs and examined immunohistochemically to determine the location of the free gelonin and the gelonin fusion construct. $VEGF_{121}$/rGel was primarily detected on endothelium of tumor (Fig. 2). In average, sixty percent of vessels positive for MECA 32 were also positive for gelonin in the group of $VEGF_{121}$/rGel – injected mice. In the tumor regions of increased vascularity (hot spots), up to 90% of tumor vessels were labeled by anti-gelonin IgG. Vessels with bound $VEGF_{121}$/rGel were homogeneously distributed within the tumor vasculature. Vessels in normal organs were unstained with the exception of the kidney where weak and diffuse staining was detected in the glomeruli. Free gelonin did not localize to tumor or normal vessels in any of the mice, indicating that only targeted gelonin was able to bind to the tumor endothelium. These results indicate that $VEGF_{121}$/rGel specifically localizes to tumor vessels, which demonstrate high density and favorable distribution of the $VEGF_{121}$/rGel – binding sites.

MDA-MB-231 Model of Experimental Pulmonary Metastases and Rationale for Therapeutic Regime

Human breast carcinoma MDA-MB-231 cells consistently lodge in lungs following intravenous injection into the tail vein of athymic or SCID mice. Micrometastases are first detected 3 to 7 days after injection of $5x10^5$ cells and macroscopic colonies develop in 100% of the injected mice within 4 to 7 weeks. Mortality occurs in all mice within 10-15 weeks. This model of experimental breast cancer metastasis examines the ability of tumor cells to

survive in the blood circulation, extravasate through the pulmonary vasculature and establish growing colonies in the lung parenchyma.

We evaluated the effect of $VEGF_{121}$/rGel on the growth and survival of the established micrometastases. We, therefore, started the treatment 8 days after injection of the tumor cells. By that time, based on our prior observations, tumor cells that were able to survive in the circulation and transverse the lung endothelial barrier are localized within the lung parenchyma and initiate tumor angiogenesis. Treatment with $VEGF_{121}$/rGel was given for the following 2 weeks as described under Methods, allowing the mice to receive the maximal tolerated accumulative dose of the drug (600 μg per mouse). Prior studies established that $VEGF_{121}$/rGel given at such dose did not cause histopathological changes in normal organs. The accumulative dose of 640-800 μg of total $VEGF_{121}$/rGel fusion protein, given i. p. over period of 4 weeks, did not induce significant toxicity as judged by morphological evaluation of normal organs. Transient loss of weight (~10%) was observed 24 hours after most of the treatments with complete weight recovery thereafter.

Colonies were allowed to expand in the absence of treatment for the three following weeks in order to evaluate long-term effect of $VEGF_{121}$/rGel on size of the colonies, proliferation index of tumor cells and their ability to induce new blood vessel formation.

Effect of $VEGF_{121}$/rGel on Number and Size of MDA-MB-231 Tumor Lesions in Lungs

Treatment with $VEGF_{121}$/rGel but not with free gelonin significantly reduced by between 42-58% both the number of colonies per lung and the size of the metastatic foci present in lung as shown in Fig. 3 and Table 1.

Effect of $VEGF_{121}$/rGel on Vascularity of the MDA-MB-231 Pulmonary Metastatic Foci.

The overall mean vascular density of lung colonies was reduced by 51% compared to the rGel treated controls (Table 2 and Fig. 4), however; the observed effect was non-uniformly distributed by tumor colony size. The greatest impact on vascularization was observed on mid-size and extremely small tumors (62 and 69% inhibition respectively) while large tumors demonstrated the lease effect (10% inhibition). The majority of lesions in the $VEGF_{121}$/rGel-treated mice (~70%) were avascular whereas only 40% of lesions from the control group did not have vessels within the metastatic lung foci.

Effect of $VEGF_{121}$/rGel on Number of Cycling Cells in the Metastatic Foci

The number of cycling tumor cells in lesions from the $VEGF_{121}$/rGel group was also reduced by ~60% as compared to controls(Fig. 5). This finding suggests that vascularity of metastases directly affects tumor cell proliferation.

Effect of $VEGF_{121}$/rGel on flk-1 Expression in Tumor Vessel Endotheluim

The expression of KDR on the remaining few vessels present in lung metastatic foci demonstrated a significant decline compared to that of lung foci present in control tumors(Fig 6). This suggests that the $VEGF_{121}$/rGel agent is able to significantly down-regulate the receptor or prevent the outgrowth of highly receptor-positive endothelial cells.

DISCUSSION

Neovascularization is a particularly important hallmark of breast tumor growth and metastatic spread *(39-43)*. The growth factor VEGF-A and the receptor KDR have both been implicated in highly metastatic breast *(44-46)*. We have previously demonstrated that the $VEGF_{121}$/rGel growth factor fusion toxin specifically targets KDR-expressing tumor vascular endothelial cells and inhibits growth of subcutaneously implanted, human tumor xenografts *(38)*. The current study was designed to evaluate its effect on development of breast cancer metastases in lungs following intravenous injection of MDA-MB-231 cells.

The salient findings of our study of the $VEGF_{121}$/rGel construct are that this fusion toxin is specifically cytotoxic to cells over-expressing the KDR receptor for VEGF. However, the human breast MDA-MB231 cells employed for these studies do not express this receptor and, therefore, were not directly affected by this agent. The antitumor effects of $VEGF_{121}$/rGel observed from our in vivo studies appear to be solely the result of targeting tumor vasculature. Administration of the $VEGF_{121}$/rGel construct to mice previously injected (i.v.) with tumor cells dramatically reduced the number of tumor colonies found in lung, their size and their vascularity. In addition, the number of cycling breast tumor cells within lung metastatic foci was found to be reduced by an average of 60%. In addition to the reduced number of blood vessels present in lung metastases of treated mice, we also found that the few vessels present had a greatly reduced expression of VEGFR2. Therefore, this construct demonstrated an impressive, long-term impact on the growth and development of breast tumor metastatic foci found in lung.

Targeting tumor vasculature with a variety of technologies has been shown to inhibit the growth and development of primary tumors as well as metastases. Recently, Shaheen, et.al. *(47)* demonstrated that small molecule tyrosine kinase inhibitors active against the receptors for VEGF, fibroblast growth factor and platelet-derived growth factors were also capable of inhibiting microvessel formation and metastases in tumor model systems. Previously, Seon et al. *(48)* demonstrated long-term anti-tumor effects of an anti-endoglin antibody conjugated with ricin A-chain(RTA) in a human breast tumor xenograft model.

Surprisingly, one finding from our study was that administration of $VEGF_{121}$/rGel resulted in a 3 fold decrease in the number of Ki-67 labeled (cycling) cells in the metastatic foci present in lung (Fig. 5). Clinical studies have suggested that tumor cell cycling may be an important prognostic marker for disease-free survival in metastatic breast cancer, but that Ki-67 labeling index, tumor microvessel density (MVD) and neovascularization appear to be independently regulated processes *(49-50)*. To our knowledge, this is the first report of a significant reduction in tumor labeling index produced by a vascular targeting agent.

Another critical finding from our studies is the observation that the vascular-ablative effects of the $VEGF_{121}$/rGel fusion construct alone were unable to completely eradicate lung metastases. Although the growth of larger pulmonary metastases was completely inhibited by this therapeutic approach, development of small, avascular, metastatic foci within lung tissue was observed. This data strongly suggests that combination of vascular targeting agents with chemotherapeutic agents or with radiotherapeutic agents which directly damage tumor cells themselves may provide for greater therapeutic effect. Studies of several vascular targeting

agents in combination with chemotherapeutic agents have already demonstrated a distinct in vivo anti-tumor advantage of this combination modality against experimental tumors in mice *(51)*. Studies by Pedley et al. *(52)* have also suggested that combination of vascular targeting and radioimmunotherapy may also present a potent antitumor combination. Finally, studies combining hyperthermia and radiotherapy with vascular targeting agents have demonstrated enhanced activity against mammary carcinoma tumors in mice *(53)*. Studies in our laboratory combining $VEGF_{121}$/rGel and various chemotherapeutic agents, biological agents or therapeutic agents targeting tumor cells are currently ongoing.

The presented findings demonstrate that $VEGF_{121}$/rGel can clearly and specifically target KDR expressing tumor vasculature both in vitro and in vivo and that this agent can have an impressive inhibitory effect on tumor metastases. Studies are continuing in our laboratory to examine the activity of this agent alone and in combination against a variety of orthotopic and metastatic tumor models.

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Figure Legends

Figure 1. VEGF$_{121}$/rGel is not cytotoxic to MDA-MB-231 cells.

Figure 1a. Western analysis demonstrating the presence of VEGFR2 on endothelial cells transfected with the R2 receptor(PAE/KDR) but not on cells expressing the FLT-1 receptor(PAE/FLT-1, negative control). As shown, the MDA-MB231 cells did not express detectable amounts of VEGFR-2.

Figure 1b. Log-phase MDA-MB231 cells were treated with various doses of VEGF$_{121}$/rGel or rGel for 72 hrs. The cytotoxic effects of both agents were similar demonstrating no specific cytotoxicity of the fusion construct compared to free toxin on these cells.

Figure 2. VEGF$_{121}$/rGel localizes to blood vessels of MDA-MB-231 tumor.

Mice bearing orthotopically-placed MDA-MB231 tumors were administered one dose (i.v., tail vein) of VEGF$_{121}$/rGel. Four hours later, the mice were sacrificed and tumors excised and fixed. Tissue sections were stained for blood vessels using the Meca 32 antibody (red) and the section was counter-stained using an anti-gelonin antibody(green). Co-localization of the stains(yellow) demonstrate the presence of the VEGF$_{121}$/rGel fusion construct specifically in blood vessels and not on tumor cells.

Figure 3. VEGF$_{121}$/rGel reduces number of large colonies in the metastatic lungs.

The size of tumor colonies was analyzed on slides stained with 6w/32 antibody which specifically recognizes human HLA antigens. The antibody delineates colonies of human tumor

cells and defines borders between metastatic lesions and mouse lung parenchyma. The largest size differences between $VEGF_{121}$/rGel and control groups were found in groups of colonies having diameter either less than 50 μm or more than 1000 μm. In the $VEGF_{121}$/rGel-treated mice more than 40% of total foci were extremely small (< 50 micron) as compared to 18% in the control group. The control mice had approximately 8% of the extremely large colonies (>1000 μm) whereas $VEGF_{121}$/rGel-treated mice did not colonies of this size.

Figure 4. $VEGF_{121}$/rGel inhibits vascularization of MDA-MB-231 pulmonary metastases.

Figure 4a. Lungs derived from $VEGF_{121}$/rGel and rGel - treated mice were stained with MECA 32 antibody and the number of vessels per mm^2 within the metastatic foci was determined. The mean number of vessels per mm^2 in lung metastases of $VEGF_{121}$/rGel - treated mice was reduced by approximately 50% as compared to those in rGel - treated mice.

Figure 4b. Representative images demonstrating reduction of vascular density in foci of comparable size in mice treated with rGel (left) and $VEGF_{121}$/rGel fusion protein (right).

Figure 5. $VEGF_{121}$/rGel inhibits proliferation of metastatic MDA-MB-231 cells in the lungs.

Frozen sections of lungs derived from $VEGF_{121}$/rGel and rGel - treated mice were stained with Ki-67 antibody. Stained sections were examined under x 40 objective to determine a number of tumor cells with positive nuclei (cycling cells). Positive cells were enumerated in 10 colonies per slide on six sections derived from individual mice per each treatment group. The mean number per group ± SEM is presented. $VEGF_{121}$/rGel treatment reduced the average number of cycling cells within the metastatic foci by approximately 60%.

Fig. 6. Detection of VEGFR2 on vasculature of metastatic lesions by anti-VEGFR2 antibody, RAFL-1. Frozen sections of lungs from mice treated with $VEGF_{121}$/rGel or free gelonin stained with monoclonal rat anti-mouse VEGFR2 antibody RAFL-1 (10 μg/ml). RAFL-1 antibody was detected by goat anti-rat IgG-HRP, as described under Methods. Sections were developed with DAB and counterstained with hematoxylin. Representative images of lung metastases of comparable size (700-800 μm in the largest diameter) from each treatment group are shown. Images were taken with an objective of X20. Note that the pulmonary metastases from the $VEGF_{121}$/rGel treated group show both reduced vessel density and decreased intensity of anti-VEGFR2 staining, as compared to control lesions.

Table 1. Effect of $VEGF_{121}$/rGel on number and size of pulmonary metastases of MDA-MB- 231 human breast carcinoma cells.

Parameter	Treatment [a] rGelonin	$VEGF_{121}$/rGel	% inhibition *vs* rGelonin treatment	P value [b]
No. surface colonies per lung (range) [c]	53.3 ± 22 (33-80)	22.4 ± 9.2 (11-41)	58.0%	0.03
No. intraparenchymal colonies per cross-section (range) [d]	22 ± 7.5 (18-28)	12.8 ± 5.5 (5-18)	42.0%	0.02
Mean area of colonies (μm) [e]	415 ± 10	201 ± 37	51.9%	0.01
Mean % of colonies-occupied area per lung section [f]	57.3 ± 19	25.6 ± 10.5	55.4%	0.01

[a] Mice with MDA-MB-231 pulmonary micrometastases were treated i.p. with $VEGF_{121}$/rGel or free gelonin as described under Methods and Results.

[b] P value was calculated using t-Student test.

[c] Lungs were fixed with Bouin's fixative for 24 hours. Number of surface white colonies was determined for each sample and averaged among 6 mice from $VEGF_{121}$/rGel or rGel control group. Mean number per group ± SEM is shown. Numbers in parentheses represent range of colonies in each group.

[d] Frozen sections were prepared from metastatic lungs. Sections were stained with 6w/32 antibody recognizing human tumor cells. Number of intraparenchymal colonies identified by brown color was determined for each cross-section and averaged among 6 samples of individual mice from $VEGF_{121}$/rGel or rGel control group. Mean number per group ± SEM is shown. Numbers in parentheses represent range of colonies in each group.

[e] Area of foci identified by 6w/32 antibody was measured by using Metaview software. Total number of evaluated colonies was 101 and 79 for rGel and $VEGF_{121}$/rGel group, respectively. Six individual slides per each group were analyzed. The mean area of colony in each group ± SEM is shown.

[f] The sum of all regions occupied by tumor cells and the total area of each lung cross-section was determined and % of metastatic regions from total was calculated. The values obtained from each slide were averaged among 6 samples from $VEGF_{121}$/rGel or rGel control group. The mean % area occupied by metastases from total area per group ± SEM is shown.

Table 2. Effect of $VEGF_{121}$/rGel on vascularity of pulmonary metastases of MDA-MB- 231 human breast carcinoma cells.

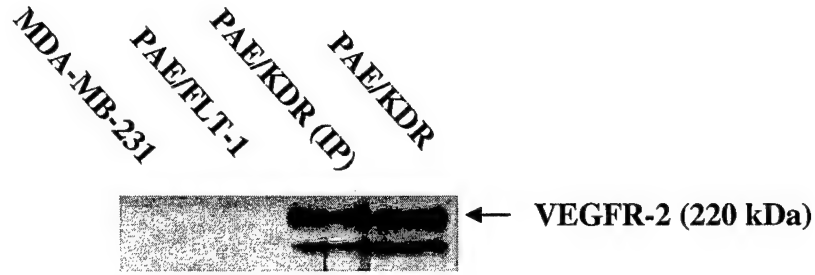
Size of colonies			No. vascularized colonies from total analyzed (%)[a]	
Group[b]	Description	Largest diameter range (μm)	rGel	$VEGF_{121}$/rGel
A	Extremely small	<50	7/24 (29%)	3/32 (9.3%)
B	Small	50-200	19/48 (39.5%)	6/24 (25%)
C	Mid-size	200-500	25/30 (83.3%)	8/25 (32%)
D	Large	500-1000	17/17 (100%)	10/11 (90.0%)
E	Extremely large	>1000	8/8 (100%)	N/A
No. vascular foci/ total analyzed (%)[c]			76/127 (59.8%)	27/92 (29.3%)

[a] Frozen lung sections from $VEGF_{121}$/rGel and rGel treated mice were stained with MECA 32 antibody. A colony was defined as vascularized if at least one blood vessel branched out from the periphery and reached a center of the lesion. Six slides per each group derived from individual mice were analyzed and data were combined.

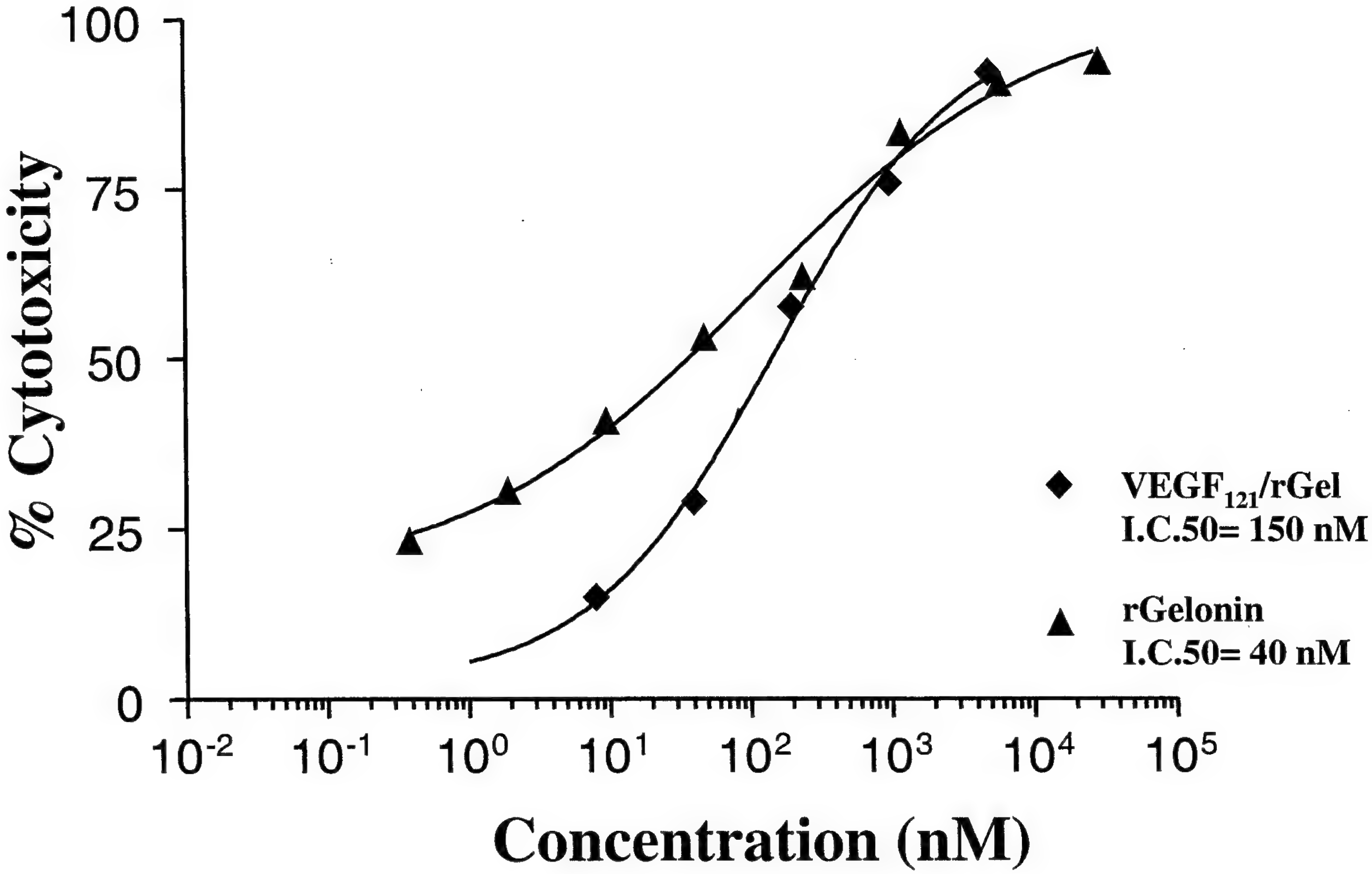
[b] Colonies identified on each slide of a metastatic lung were subdivided into 5 groups (A-E) according to their largest diameter.

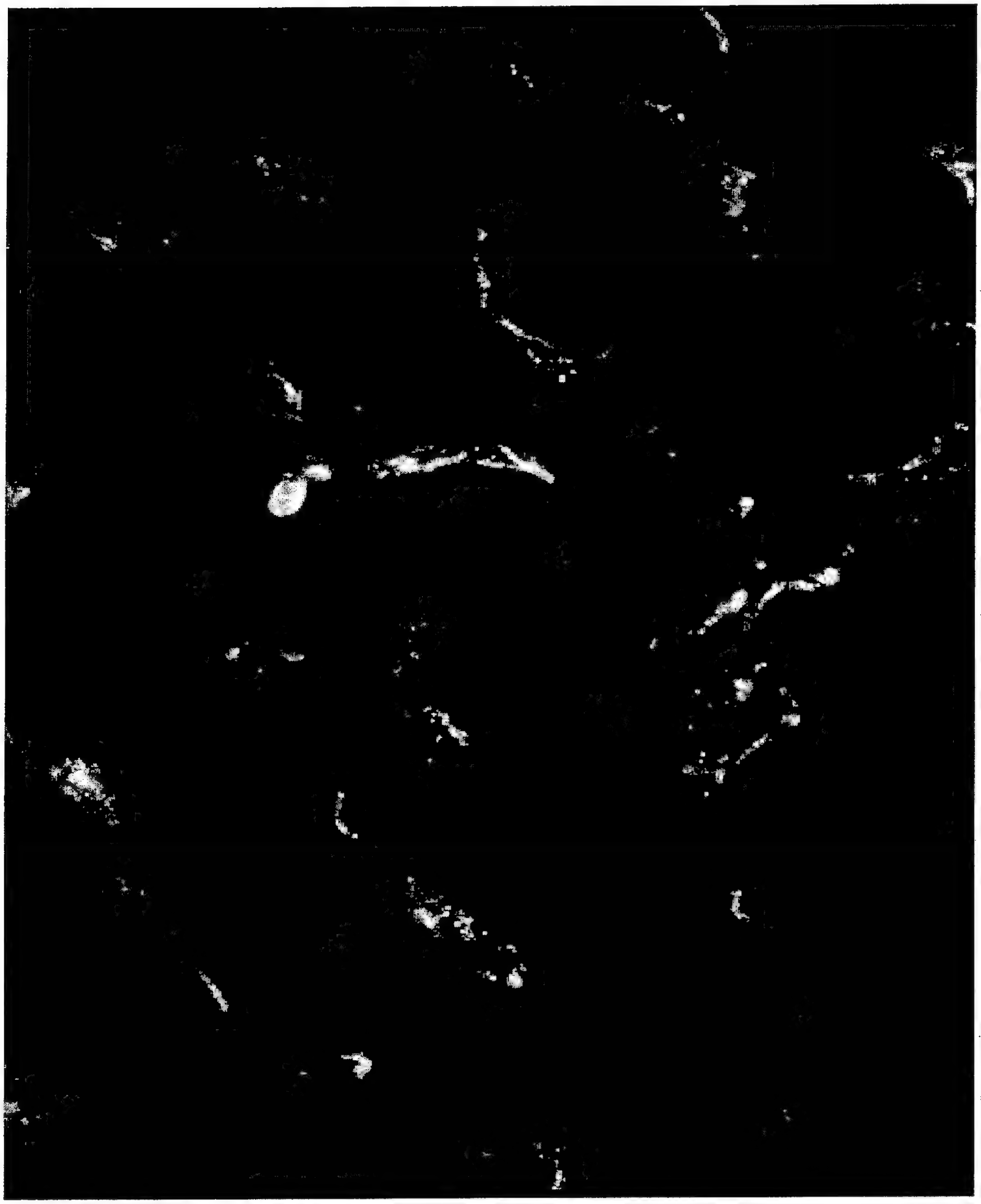
[c] Total number of the analyzed colonies was 127 and 92 for rGel and $VEGF_{121}$/rGel treated groups, respectively. Seventy percent of foci in the $VEGF_{121}$/rGel-treated group were avascular whereas only 40% of lesions from the control group did not have vessels within the metastatic foci.

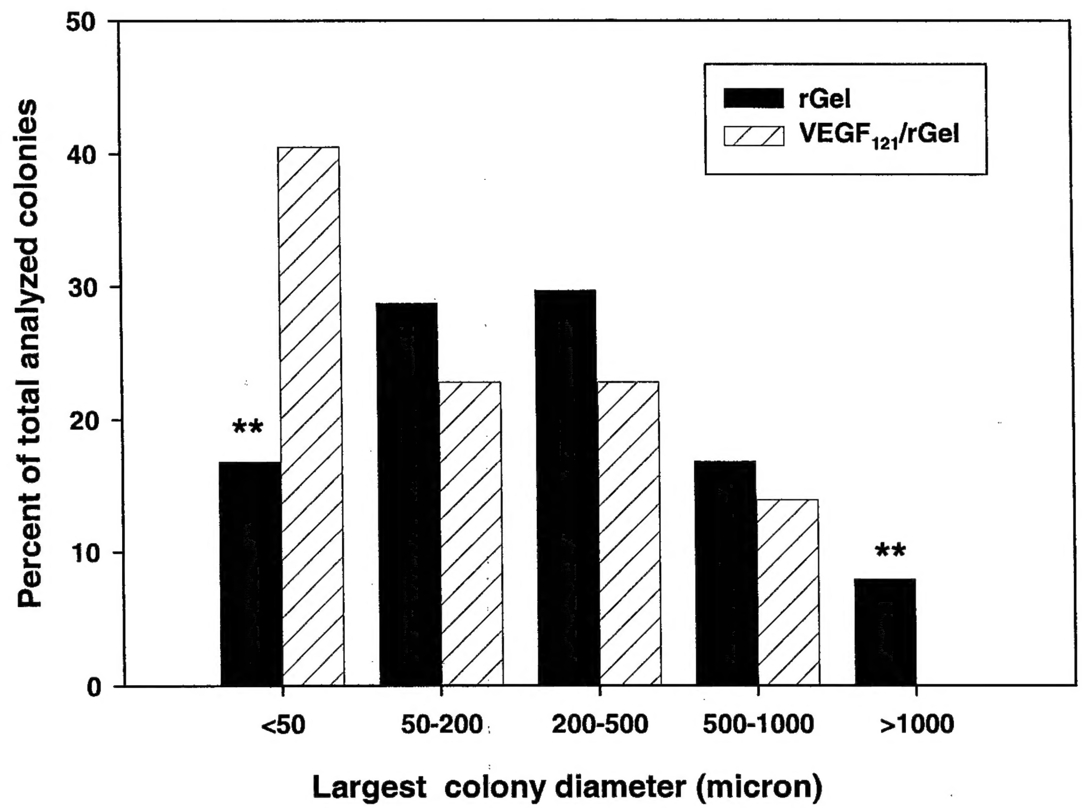
A.


MDA-MB-231
PAE/FLT-1
PAE/KDR (IP)
PAE/KDR
VEGFR-2 (220 kDa)

B.


100
75
50
25
0
% Cytotoxicity
10^{-2} 10^{-1} 10^{0} 10^{1} 10^{2} 10^{3} 10^{4} 10^{5}
Concentration (nM)
$VEGF_{121}$/rGel
I.C.50= 150 nM
rGelonin
I.C.50= 40 nM

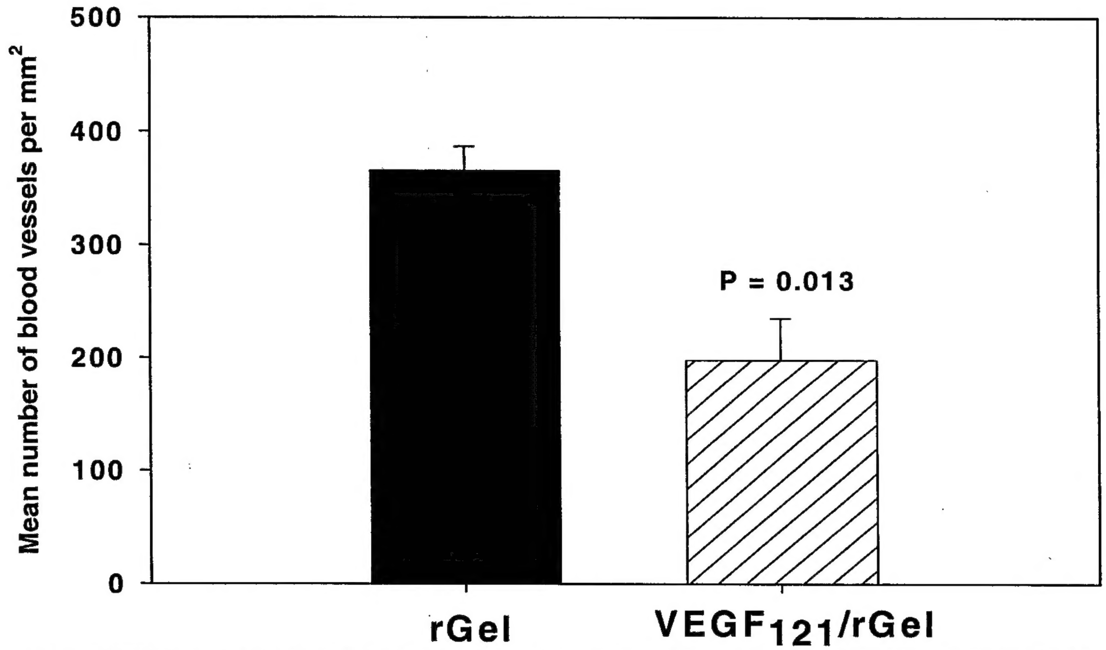




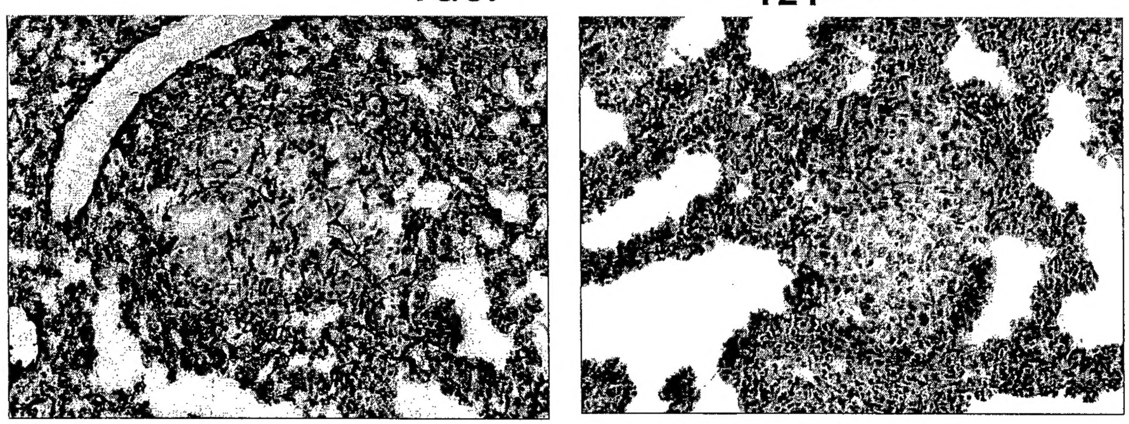
50
40
30
20
10
0
Percent of total analyzed colonies
rGel
VEGF121/rGel
**
**
<50
50-200
200-500
500-1000
>1000
Largest colony diameter (micron)

Effect of $VEGF_{121}$/rGel on vascular density of metastatic foci in lungs

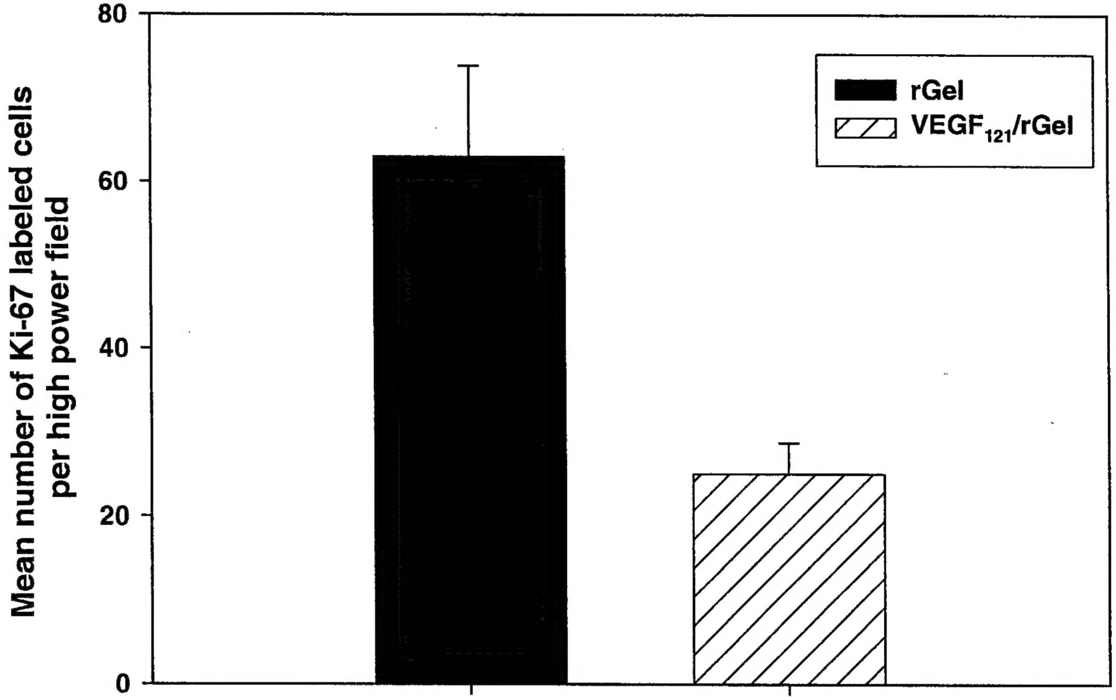
A



B



Effect of $VEGF_{121}$/rGel treatment on proliferation of metastatic cells in lungs



Control

VEGF$_{121}$/rGel